Vol. I. No. 5] [October, 1920.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

Editors:

C. H. BROWNING
C. L. EVANS
P. FILDES
W. E. GYE
E. L. KENNAWAY
W. H. KETTLE
J. McINTOSH
J. A. MURRAY
W. J. TULLOCH
C. M. WILSON

With the Support of:

F. W. ANDREWES (London)
W. M. BAYLISS (London)
W. BULLOCH (London)
S. L. CUMMINS (London)
H. H. DALE (London)
A. M. DRENNAN (Otago, N.Z.)
J. W. H. EYRE (London)
LEONARD FINDLAY (Glasgow)
DUNCAN GRAHAM (Toronto)
R. T. HEWLETT (London)
F. G. HOPKINS (Cambridge)
W. B. LEISHMAN (London)
J. J. MACKENZIE (Toronto)
T. J. MACKIE (Cape Town)
H. OSTEL (Montreal)
LEONARD ROGERS (Calcutta)
C. S. SHERRINGTON (Oxford)
A. E. WRIGHT (London)

LONDON: H. K. LEWIS & CO. LTD., 136, GOWER STREET, W.C. 1.

EDITORIAL AND PUBLISHING OFFICE: 28, GOWER PLACE, W.C. 1.

CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO.

Annual Subscription, post free, Two Pounds net.

Single Numbers, Seven and Sixpence net.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

EDITORIAL NOTICE TO CONTRIBUTORS.

Scope of Papers.—This Journal has been founded for the publication of original communications describing the technique and results of experimental researches into the causation, diagnosis and cure of disease in man. Papers upon bacteriological, biochemical, physiological, serological and other subjects are therefore acceptable when bearing directly upon these objects.

A section will be devoted to short communications upon new technical methods applicable to those subjects which come within the scope of the Journal.

It is also the intention of the Editors to publish occasional "critical reviews" upon pathological subjects which may at the time be in need of critical survey. Such reviews may be of value to workers who are not in touch immediately with considered opinions upon the questions discussed.

Forwarding Papers.—Papers should be addressed to—

THE EDITORS,
British Journal of Experimental Pathology,
28, GOWER PLACE,
LONDON, W.C. 1,

or to any of the Editors personally.

Date of Publication.—The Journal is published bi-monthly, in February, April, June, August, October, and December.

Priority of Publication.—Papers accepted for publication will be printed as far as possible in the order of their receipt by the Editors, but those sent from overseas will receive priority in publication according to the date of posting instead of the date of receipt.

Conditions of Publication.—Contributors should prepare their manuscripts with the greatest attention to detail. The fact of submitting a paper for publication will be taken by the Editors as an admission by the Author of his liability to pay for all unreasonable alterations made by him in proofs, and for mistakes due to lack of clearness in the preparation of the manuscript.

Illustrations and charts will be accepted for publication, but if, in the opinion of the Editors, they are not required to increase the clarity of the argument, they must be paid for by the Authors.

References.—Authors are requested to prepare their references according to a systematic plan, namely, as given in the *Index Catalogue of the Surgeon-General's Library* at Washington. They should be numbered consecutively in the text, and collected at the end of the paper in the same order under "References." The initials, title, year, volume and page should all be included.

Proofs.—To avoid delay, proofs will not be submitted to Authors overseas unless they express a desire to see them, or unless the Editors think it necessary. Overseas Authors are therefore particularly reminded of the importance of preparing their manuscripts in such a way that error need not arise.

Reprints.—25 reprints will be supplied free to authors. Additional copies must be ordered from the Publishers *before the issue is printed*, and will be charged for according to the following scale:

		£ s. d.	£ s. d.	£ s. d.	£ s. d.
Number of Additional Reprints	25	1–4 pp., 0 11 6	5–8 pp., 1 2 6	9–12 pp., 1 13 6	13–16 pp., 2 4 6
	50	„ 0 13 9	„ 1 6 6	„ 1 19 0	„ 2 12 0
	75	„ 0 16 0	„ 1 10 6	„ 2 4 6	„ 2 19 6
	100	„ 0 18 6	„ 1 14 6	„ 2 10 0	„ 3 7 0

Authors working in countries where the rates of exchange are unfavourable will be charged for reprints according to pre-war rates (25 francs = 20 shillings).

THE BRITISH JOURNAL
OF
EXPERIMENTAL PATHOLOGY

THE VALUE OF SIMULTANEOUS TESTING FOR THE WASSERMANN REACTION, WITH TWO DIFFERENT ANTIGENS AND THE "ICE-BOX METHOD."

T. J. MACKIE AND C. C. ROWLAND.
From the Bacteriological Department, University of Cape Town.

Forwarded for publication June 8th, 1920.

THE failure of the Wassermann reaction to detect even under optimum conditions a proportion of syphilitic cases, especially those in the early primary, tertiary and latent stages, undoubtedly represents a practical limitation which detracts considerably from the diagnostic application of the test. While the sera of many such cases show no actual difference in the Wassermann test from normal sera, it must be recognised that a weakly reacting serum may, under certain conditions, escape recognition and yield a negative result. Variations in deviability of complement may be responsible for quantitative differences in Wassermann effects, and a serum which is weakly positive with one specimen of complement may react negatively with a complement of lesser deviability. Apart from this factor, the Wassermann reaction is subject to other influences, and any procedure which adds to the sensitiveness of the test without eliciting non-specific positive results must be regarded as of the greatest practical value for the detection of sera which are likely to yield even under suitable conditions only a weak reaction.

SIMULTANEOUS TESTING WITH TWO ANTIGENS.

That the results depend among other factors on the type of antigen used has been indicated by the observations of many workers on this subject, and while an alcoholic heart extract *plus* cholesterol has proved highly satisfactory and has been much advocated, it has been shown by Browning and Kennaway (1) that the greater sensitiveness of this antigen is by no means a constant,

and that with certain sera a liver lecithin *plus* cholesterol antigen may elicit positive results when the heart extract is inactive in antigenic property. Thus, if only one of these antigens is used a proportion of weakly positive reactions may be missed. We have therefore applied in a series of cases the routine procedure advocated by Browning and Kennaway, in which each serum was tested simultaneously with (*a*) liver lecithin *plus* cholesterol antigen and (*b*) heart extract *plus* cholesterol antigen, using a fixed quantity of serum, and ascertaining in each case the degree of complement fixation by testing with varying doses of complement.

Method of carrying out the Reaction.

The antigens.—(*a*) The liver lecithin *plus* cholesterol antigen was prepared according to the method of Browning, Cruickshank and Mackenzie (2); (*b*) the heart extract was obtained from sheep's heart minced, and finely ground with sand, and then extracted for four days with absolute ethyl alcohol (20 grm. heart to 100 c.c. alcohol); the extract was saturated with cholesterol (about 0·4 per cent.).

The patient's serum was heated at 57° C. for half an hour, according to the usual procedure.

The hæmolytic system was ox's red corpuscles (5 per cent. suspension of blood) with a rabbit *versus* ox immune body, and guinea-pig's serum was used as complement.

Both antigens were used for the test in a 1 : 12 dilution made up in the form of a turbid suspension by slow admixture of the alcoholic solution with the diluent (0·85 per cent. NaCl).

The M.H.D. of the complement for 0·5 c.c. of the sensitised blood suspension was estimated by preliminary test.

The amounts of the different reagents were as follows:

	Test.			Antigen Control.		Serum Control.	
	1.	2.	3.	1.	2.	1.	2.
Antigen 1 : 12 .	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	0·5 c.c.	Saline 0·5 c.c. 0·85 %	0·5 c.c.
Serum . .	0·05 c.c.	0·05 c.c.	0·05 c.c.	—	—	0·05 c.c.	0·05 c.c.
Complement .	4 M.H.D.	8 M.H.D.	14 M.H.D.	2 M.H.D.	4 M.H.D.	2 M.H.D.	4 M.H.D.

The tubes were incubated for one and a half hours at 37° C., and then 0·5 c.c. of the sensitised blood suspension was added to each. The results were read after one hour's incubation at 37° C. The dilution and amounts of the antigens were determined by a large series of comparative tests with several known negative and a number of positive sera. According to Ruediger (3) higher dilutions of antigen may yield more marked reactions than lower dilutions; we have not found this to be the case in our experiments, in which dilutions ranging from 1 : 6 to 1 : 500 were tested, and therefore the largest amount of antigen which constantly failed to elicit non-specific reactions or a marked anti-complementary effect by itself was selected for routine testing. These tests were carried out with a number of different specimens of guinea-pig's complement, and it was generally found that the optimum amount of both antigens thus determined was 0·5 c.c. of a 1 : 12 dilution (using 0·05 c.c. of patient's serum).

It was noted in the case of certain sera (from treated cases) that amounts of serum less than 0·05 c.c. failed to yield a positive result; this amount of serum never elicited non-specific

reactions. Larger quantities in some cases showed marked anti-complementary effects, so that this amount was selected as a likely optimum.

On testing by this method a number of known negative and weakly positive sera (from treated syphilitic cases which had previously yielded a strongly positive reaction) with different specimens of guinea-pig's complement, which of course varies considerably in deviability, it was found that in the case of both antigens with negative sera 4 M.H.D. (tube 1) was the smallest amount of complement which constantly produced "almost complete" or "complete" lysis, provided the antigen and serum were not excessively inhibitory by themselves as shown by the respective controls. At the same time the weakly positive sera with one or both antigens absorbed this amount of complement, and no lysis or only a trace of lysis occurred in the first tube. Thus the occurrence of complete or almost complete inhibition of lysis in the first tube was regarded as a criterion of a positive reaction, provided that negative control sera (which were always included in each series of tests) showed complete or almost complete lysis in this tube (see tables). Of course, in the majority of positive reactions the deviation is more marked.

A series of 300 sera were tested with this technique and using two antigens, with the following results:

1. *Negative* with both antigens	138		
2. *Positive* with both antigens, and showing an equal degree of complement-deviation with both	81	50 %	of the total positive reactions.
3. *Positive* with both antigens, but showing more marked complement-deviation with the heart cholesterol antigen	34	20·98 %	ditto.
4. *Positive* with both antigens, but showing more marked complement-deviation with the lecithin cholesterol antigen	17	10·49 %	ditto.
		81·47 %	ditto.
5. *Positive* with the lecithin cholesterol antigen, but *negative* with the heart cholesterol antigen . .	11	6·79 %	ditto.
6. *Positive* with the heart cholesterol antigen, but *negative* with the lecithin cholesterol antigen	19	11·72 %	ditto.
		18·51 %	ditto.

Note.—"Positive" includes also "weak positive" reactions.

Of the positive results 81·4 per cent. yielded definite reactions with both antigens; 50 per cent. showed an equal degree of complement-deviation with the two antigens, while 20·9 per cent. gave a more marked result with the heart antigen and 10·4 per cent. a more marked effect with the liver lecithin antigen. Thus the heart antigen was usually the more sensitive, but not invariably so; 11·7 per cent. gave a positive reaction with this antigen, while the same sera reacted negatively with the lecithin antigen; on the other hand 6·7 per cent. reacted positively with the lecithin antigen while the heart extract gave a negative result. About 18·5 per cent. of all the positive results belonged to this category in which a positive result was obtained with one antigen while a negative effect occurred with the other. The 11 cases belonging to class 5 comprised 6 early primary cases, 4 treated cases, and 1 case of aortic aneurysm. The cases in class 6 were: 7 early primary cases, 11 cases which had received antisyphilitic treatment, and 1 case of iritis with

a syphilitic history. Accordingly it was possible to detect cases in the early primary stage by using the two antigens, when one might fail to elicit a positive result if only a single antigen were used. It has been suggested by Walker and Swift ([4]) that the value of the heart antigen is particularly marked in testing treated cases, and while this is evident, the liver lecithin cholesterol antigen is also capable of detecting a certain proportion of such cases which do not react positively with the heart antigen.

The Table shows some of these results. In series 1, while three sera reacted positively with the heart antigen, one serum (C) was positive only with the liver antigen. It was sometimes observed that with one complement all the positive sera reacted more strongly with one antigen than the other (series 2), but with the same specimen of complement "converse" effects were frequently noted, as shown in series 1 and 3.

Apart from the value of multiple antigens in eliciting weakly positive reactions, it frequently happens that one antigen yields a more strongly positive reaction than the other and renders the result more conclusive, and even if the results are identical the use of a second antigen supplies a valuable confirmatory test. The method is particularly valuable in primary cases and those which are being tested as a guide to treatment.

THE ICE-BOX METHOD.

In a recent report on this method of performing the Wassermann test, Fairley and Sullivan ([5]) indicated that the sensitiveness of the reaction was distinctly increased by allowing the interaction of antigen, serum and complement to occur at 8° C. for a period of six hours, as compared with the results of incubating at 37° C. by the usual method. They supposed that the value of the procedure depends essentially on the increased time allowed for complement-fixation to occur, and showed that complement-fixation takes place as readily at low temperatures as at 37° C., and that it is possible to expose complement for a long period at 8° C. without deterioration in its hæmolytic value. As this procedure seemed to suggest a possible method of increasing the sensitiveness of the reaction, we have carried out a series of 102 tests in which the effect of incubation at 37° C. for one and a half hours was compared with that of allowing the interaction to occur at 8° C. for six hours, using the two antigens referred to above in both cases. Of the 102 sera tested in this way

---

Explanation of symbols: L = Liver lecithin *plus* cholesterol. H = Heart extract *plus* cholesterol. c = complete lysis. jc = just complete. alc = almost complete. mk = marked. dst = distinct. tr = trace. o = no lysis.

Note.—The complement M.H.D. in Series 1 and 3 = 0·004 c.c.; in Series 2, 0·006 c.c.

The serum controls gave complete or just complete lysis with two doses of complement in every case. The antigen controls with two doses of complement gave just complete lysis in each series, except in the case of heart extract *plus* cholesterol in Series 1, which gave almost complete lysis. The tests of the positive and negative control sera have been omitted from Series 2 and 3.

SERIES 1.

	Antigen.	Test.	Result.
Tube	—	1 . 2 . 3	—
Doses of complement . .	—	4 . 8 . 14	—
Negative control serum .	L	jc . c . c	Negative.
	H	jc . c . c	,,
Positive control serum .	L	o . o . tr	Marked positive.
	H	o . o . tr	,, ,,
Serum A: Primary sore .	L	alc . c . c	Negative.
	H	o . tr . jc	Positive.
Serum B: Treated case .	L	alc . c . c	Negative.
	H	tr . alc . c	Weak positive.
Serum C: Treated case .	L	tr . alc . c	,, ,,
	H	jc . c . c	Negative.
Serum D: Treated case .	L	alc . c . c	,,
	H	tr . alc . c	Weak positive.

SERIES 2.

Serum A: Primary sore .	L	c . c . c	Negative.
	H	tr . alc . c	Weak positive.
Serum B: Iritis . . .	L	c . c . c	Negative.
	H	tr . alc . c	Weak positive.
Serum C: Primary sore .	L	alc . c . c	Negative.
	H	o . tr . dst	Positive.
Serum D: Secondary syphilis	L	o . tr . mk	,,
	H	o . o . o	Marked positive.
Serum E: Primary sore .	L	o . dst . c	Positive.
	H	o . o . dst	Marked positive.
Serum F: Tabes dorsalis .	L	tr . c . c	Weak positive.
	H	o . tr . c	Positive.

SERIES 3.

Serum A: Treated case .	L	tr . jc . c	Weak positive.
	H	jc . c . c	Negative.
Serum B: Treated case .	L	c . c . c	,,
	H	tr . dst . c	Positive.
Serum C: Primary sore .	L	c . c . c	Negative.
	H	tr . alc . c	Weak positive.
Serum D: Primary sore .	L	alc . jc . c	Negative.
	H	o . tr . c	Positive.
Serum E: Treated case .	L	tr . mk . c	Weak positive.
	H	alc . c . c	Negative.
Serum F: Known positive .	L	o . o . mk	Positive.
	H	o . alc . c	Weak positive.
Serum G: Known positive .	L	o . alc . c	,, ,,
	H	o . o . dst	Positive.

50 reacted negatively and 52 positively by both methods, and there was no qualitative divergence of results. As regards the comparative degree of complement-deviation by the two methods the results were as follows:

Complement-fixation by the Ice-box Method as compared with that at 37°C. by the Usual Method.

Heart antigen.	Liver lecithin antigen.	
More marked . . .	More marked . . .	3 cases.
Less marked . . .	Less marked . . .	5 ,,
Equal (approximately) .	More marked . . .	1 case.
More marked . . .	Equal (approximately) .	3 cases.
Equal (approximately) .	Less marked . . .	3 ,,
Less marked . . .	Equal (approximately) .	1 case.
Equal (approximately) .	Equal (approximately) .	36 cases.

It seems doubtful from these results if this method yields any advantages as a substitute for the original method, and it has the disadvantage of the long period required for carrying out the complete test. In certain suspicious cases, however, yielding a negative result, it might be applied for purposes of confirmation. It is also of interest to note the differences elicited between the action of the two antigens in these comparative tests.

CONCLUSIONS.

(1) The sensitiveness of the Wassermann reaction as here described can be increased, especially in early primary and treated cases, by testing simultaneously with a liver lecithin *plus* cholesterol antigen and a heart extract *plus* cholesterol antigen, *i. e.* a proportion of weakly positive reactions can be detected in this way which would escape notice if only one of these antigens were used.

(2) The ice-box modification as applied to the method described does not confer any appreciable advantage over the usual procedure of incubating the mixture of antigen, serum and complement at 37°C. for one and a half hours.

REFERENCES.

1. Browning, C. H., and Kennaway, E. L.—"A Note on the Use of 'Antigens' in Diagnosis by the Wassermann Reaction," *Lancet*, 1918, **2**, 753.
2. Browning, C. H., Cruickshank, J., and Mackenzie, I.—"Constituents concerned in the Wassermann Syphilis Reaction, with Special Reference to Lecithin and Cholesterin," *J. Pathol. and Bacteriol.*, 1910, **14**, 484.
3. Ruediger, E. H.—"The Influence of Antigen Dilution on the Wassermann Reaction," *J. Infect. Dis.*, 1919, **25**, 225.
4. Walker, I. C., and Swift, H. F.—"A Study of the Addition of Cholesterin to the Alcoholic Extracts of Tissues for Antigens in the Wassermann Reaction," *J. Exper. Med.*, 1913, **18**, 75.
5. Fairley, N. H., and Sullivan, C. F.—"A Preliminary Report on the Ice-box Method of Performing the Wassermann Reaction," *J. R. Army Med. Corps*, 1919, **33**, 268.

VARIATIONS IN THE BENZIDINE PEROXIDASE REACTION DEPENDING ON FIXATIVE, PHYSIOLOGICAL ACTIVITY AND TYPE OF ANIMAL.

M. L. MENTEN.

From the Pathological Laboratories, University of Pittsburgh, Pittsburgh, Pa.

Received for publication July 27th, 1920.

In 1909, Schultze (1) discovered that when films of blood were immersed in solutions of α-naphthol and dimethylparaphenylenediamine these substances were oxidised by contact with cells of myelogenous origin, as was shown by the development within them of coloured granules. Cells of lymphatic origin, lacking this oxidising property, remained uncoloured under the same conditions. The demonstration that the difference in the composition of the cells of these two series was sufficiently marked to influence either favourably or unfavourably the oxidation of adsorption complexes of phenolic dye substances within the leucocytes led to a further study of many phenol derivatives similar to α-naphthol. Among the more recently investigated of these phenols is benzidine, whose first employment in the microchemistry of the blood is accredited to Fischel.(2) However, the use of this base as a microchemical agent for the demonstration of the so-called peroxidases has not always yielded uniformly positive results in the hands of the investigators who have reported upon its use. Among the undesirable effects which have been obtained are staining of elements other than the myelogenous cells, the transient character of the colour formed, and the difficulty in finding a suitable counterstain. Indeed, so unsatisfactory did Fischel (2) find benzidine that he was led to substitute for the base the benzidine sodium monosulphate. However, Fiessinger and Rudowska (3), Loele (4) and Graham (5) each successfully developed with benzidine (base) a technique, by means of which the cells of the myelogenous series could be readily differentiated from the lymphocytes though the colour-formation was not identical in the different methods used. Thus Fiessinger and Rudowska (3), after immersing blood-films in a 2 per cent. solution of benzidine in absolute alcohol and then exposing them to solutions of hydrogen peroxide, obtained blue granules. Graham (5), treating blood films with a 0·5 per cent. solution of benzidine in 75 per cent. alcohol to which was added 0·2 per cent. hydrogen peroxide, found brown granules in neutrophiles and eosinophiles.

Alcoholic benzidine to which hydrogen peroxide has been added is a reagent in which a series of oxidation changes may be induced by exceedingly slight modifications in the solvent. The fact that different colours may be produced in the benzidine taken up by the polymorphonuclear leucocytes suggests that these colour differences in the cells may be due either to variations in the composition or in the physico-chemical state of these blood elements.

Our attention was first directed to such possible variations in the course of some experiments in which benzidine was employed as one of the methods of demonstrating the peroxidase content of the leucocytes in blood from experimental guinea-pigs. In using the Graham solution outlined above and carefully following the technique recommended we failed to duplicate the blood-picture described by the author. Only a small percentage of the polymorphonuclear leucocytes of the guinea-pig contained yellow granules. Further investigation revealed the fact that an alcoholic solution of benzidine, both with and without the addition of hydrogen peroxide, would not only reveal differences in the peroxidase content between cells of myelogenous and lymphatic origin, but by slightly modifying the concentration of the various constituents of the solution, variations in the intrinsic composition of the neutrophilic leucocytes from different animals could further be distinguished. Only neutrophiles and eosinophiles of human blood give the results reported by Graham. The benzidine reagent recommended by that author is not specific for all myelogenous cells from the blood of lower animals. (We have not had the opportunity of comparing the benzidine reaction in leucocytes of human blood with that of the white blood-cells from the other families of the Anthropoidea more nearly related to man. It would seem probable that blood elements from some of the genera of the Simiidæ might give reactions similar to those of man.)

Besides differences in composition of cells peculiar to various animals, physiological activity and fixation were modifying factors which contributed to variations in the leucocyte reaction. Results of the studies dealing with these various factors are outlined in the following pages.

The solution used in the first series of experiments was as nearly as possible that described by Graham, and consisted, as already stated, of a 0·5 per cent. solution of benzidine in 75 per cent. alcohol, to which was added 0·2 per cent. hydrogen peroxide. In the early experiments the hydrogen peroxide and the benzidine were added to the alcohol at the time of preparation and the solution was then allowed to stand for forty-eight hours or longer. In the later experiments, and especially in the study of pus, it was found more convenient to make up a stock solution of alcoholic benzidine and to add the hydrogen peroxide immediately before the staining of the preparations. The films, after being thoroughly washed in distilled water, were counterstained with a saturated solution of thionin in 75 per cent. alcohol, to

every 10 c.c. of which was added 2 drops of a 2 per cent. solution of sodium carbonate immediately before using. The films were then mounted in 50 per cent. aqueous glycerine.

The benzidine consisted of two Merck preparations. One was a white crystalline salt and the other was a fine greyish-mauve powder. No appreciable difference could be distinguished between the results obtained by either substance. We did not note that the mauve powder gave a more diffuse reaction as described by Graham. The hydrogen peroxide was U.S.P., neutral in reaction, and contained $\frac{1}{6}$ gr. of acetanelid to the fluid ounce. While the reaction could be readily obtained with freshly prepared benzidine solutions the formation of colour was somewhat accelerated when this had been prepared one or two days previously, during which time it acquired a brown colour due to oxidative changes. In order to determine the time of immersion required to give the optimal results the time of staining was varied from five minutes to seven hours, but as in practically every instance a well-marked colour was developed in fifteen minutes this was arbitrarily chosen as the exposure time for all films studied. Only blood-cells were studied. To compare the benzidine reaction in blood of different animals unfixed films were used and these were dried in air. They must be thin and evenly spread, as the colour which develops depends somewhat on the thickness of the film. Freshly made films were used, although no appreciable difference in the intensity of the colour could be detected if these were kept for two or three weeks provided they were not exposed to the action of light. As it was noted that fixation was not a negligible factor in the development of colour the effect of treatment with 5 per cent. Schering's formalin and with absolute alcohol previous to staining with benzidine was studied. The results are noted in the appended tables.

Blood was taken from human patients and from the following animals: white rat, white mouse, guinea-pig, dog, rabbit and hen.

When one treats films of blood from the above animals with the Graham solution of benzidine and compares them with similarly treated films of human blood, one observes, in agreement with the findings of the majority of the other workers in this field, that the lymphocytes in all of the specimens are invariably negative. The reaction in the cells of the myelogenous series is not uniformly positive, but varies with the type of animal and with the varying concentrations of alcohol and of hydrogen peroxide used. The granules of all of the human polymorphonuclear leucocytes are invariably stained a yellowish-brown, while in those of the lower animals these either remain completely negative or only a small percentage stain. In five guinea-pigs, counts of the polymorphonuclear leucocytes were made, and the estimated percentages of positive cells among these were 8·7, 10·4, 11·6, 15 and 20·5. These figures vary within fairly wide limits, and while these variations are no doubt partly due to the method of preparing the films, they can be largely accounted for by

differences in the blood of the individual animals. Mouse and rat showed similar variation. In some of the rabbits studied, and especially the younger ones, the yellow colour in the granules was very pale at the end of fifteen minutes' staining, and a well-defined reaction could be obtained only by prolonging the immersion to thirty or forty minutes. In all of these animals, if the hydrogen peroxide was increased to 1 per cent., all of the cells except an occasional polymorphonuclear leucocyte were negative. However, if the same benzidine solution free from hydrogen peroxide was used then the percentage of positively reacting cells rose to a 100. Adding hydrogen peroxide in progressively increasing quantity decreased the number of positively reacting cells, which became almost completely negative at 1 per cent. In the few cells showing yellow granules at this concentration the tendency to retain this colour is very marked. Colour-formation in these cells is only inhibited by increasing the hydrogen peroxide to a high concentration. Both the size of this cell and its contained granules are larger than normal. A greenish cast was often observed in the negatively reacting cells.

The blood of the hen was interesting, since leucocytes containing the rod-shaped granules were negative not only in solutions free from hydrogen peroxide, but also in those to which 0·2 and 1·0 per cent. hydrogen peroxide had been added. In the first solution all of the polymorphonuclear leucocytes containing the round granules were positive. At 0·2 per cent. the number had decreased to about a half, and at 1 per cent. all of the cells with round, coloured granules were practically absent. The thrombocytes were negative in all three solutions. In the erythrocytes a definite yellow reaction occurred when 1 per cent. hydrogen peroxide was present in the reagent, and when the concentration of this substance reached 1·2 per cent. brown granules were found lying around the nucleus.

If the slides already described were re-examined four or five weeks after they were originally stained, the nuclear thionin staining had disappeared and the brown "peroxidase" colour was much more evident. This is probably due to the continued oxidation of the benzidine compounds which have been adsorbed by the cell. These preparations are readily re-stained with thionin.

As the percentage of hydrogen peroxide is increased the yellow reaction is also observed in the erythrocytes, but it was not found in a concentration below 0·4 per cent. It also occurs by prolonging the time of immersion, thus confirming Graham's (5) observation. Only an occasional cell exhibiting granules stained with thionin was found in any of the preparations.

While minor differences exist between the blood from different orders, the most marked difference was found between man and the order next below. Only in man and dog have these variations been studied in detail. The concentrations of alcohol used in these studies were 45, 75, 85, 90, 95 and absolute. In all except the first the concentration of benzidine was 0·5 per cent. In 45 per cent. alcohol benzidine is not soluble to this extent and a

saturated solution was therefore used. Smears were stained in each of the following solutions, viz. one free from alcohol, and the other two containing hydrogen peroxide in the amounts of 0·2 and 1·0 per cent. Films made at the same time were not only dried in air but also fixed in 5 per cent. formalin and absolute alcohol, and these three series of slides were then subjected to the same technique. The results have been arranged in Tables I and II (pages 230–231).

There are several points of interest in these tables. Perhaps the most striking feature is the fact that the human smears, both with and without fixation, are not coloured by the benzidine solutions unless hydrogen peroxide has been added. No colour is developed in human blood-cells in alcoholic benzidine. It is also interesting to note that unfixed human cells respond much less readily to the action of benzidine than the unfixed cells from the blood of the lower animals.

Formalin treatment previous to staining enhances the effect of the benzidine in all the bloods examined when the higher concentrations of alcohol are used. Thus the highest concentration of alcoholic benzidine in which films of untreated human blood stain is 85 per cent., while following formalin fixation positive results can be obtained with 95 per cent. alcoholic solutions. However, when 75 and 45 per cent. alcohol are employed as solvents the efficiency of the benzidine as a staining reagent is diminished. The intensity of the resulting reaction when hydrogen peroxide is used depends largely upon the strength of the alcohol and consequently upon the degree of dissociation of the benzidine.

In the lower animals the characteristic yellow staining in leucocytes can be readily obtained with benzidine solutions which are free from hydrogen peroxide. With fixed preparations in the 75 and 85 per cent. alcoholic solutions there is a marked tendency for the formation of dark blue acicular crystals to occur. While the crystals appear to arise from material which has been extruded from the cell this is difficult to decide definitely. The loss of the granular character of the yellow-brown reaction may be met with not uncommonly in the lower concentrations of alcohol after formalin fixation, and in these cases the colour is reduced in intensity and assumes a pale yellow cast. The colour may be found in all shades, from a pale yellow to a deep brown. This coloured material, except where the granular aspect is lost, lies on the periphery of the granule, and undoubtedly is of the character of an adsorption compound similar to that described by us [8] and found with aqueous solutions of α-naphthol and paraphenylenediamine.

With negative results the cells frequently possess a very pale greenish-yellow cast. The action of formalin fixation, as is to be observed from the tabulated results, differs somewhat from that of absolute alcohol. With the latter less divergence from the colour picture obtained in unfixed films is observed. As previously indicated, only in thin, evenly-spread films are uniform clear-cut results found. Where the cells lie closely packed the staining may be diffuse and the colour then varies considerably.

TABLE I.—*Blood from Human Patient.*

0·5 per cent. benzidine is contained in each of the following solutions.	Films unfixed, dried in air.	Films fixed in 5 per cent. formalin.	Films fixed in absolute alcohol.
Absolute alcohol	Negative except for 1 or 2 cells	Negative except for 1 or 2 cells	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	Ditto	„ „ „	Ditto.
„ + 1·0 per cent. H_2O_2	„	„ „ „	„
95 per cent. alcohol	Negative except for 1 or 2 cells	All cells contain yellow granules { in large cells colour ++++ / „ small „ „ +++ }	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	Ditto	Ditto { „ large „ „ +++ / „ small „ „ ++ }	Ditto.
„ + 1·0 per cent. H_2O_2	„	„ { „ large „ „ ++ / „ small „ „ + }	„
90 per cent. alcohol	Negative except for 1 or 2 cells	All cells contain yellow granules and cells are surrounded by deep blue acicular crystals	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	Ditto	All cells contain yellow granules less intense in colour and granules present	50 per cent. of cells contain yellow granules.
„ + 1·0 per cent. H_2O_2	„	All cells contain pale granules and deep blue acicular crystals surround the cells	5 per cent. of cells contain yellow granules.
85 per cent. alcohol	Negative except for 1 or 2 cells	All cells negative. Few blue crystals surround the cells	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	All cells contain yellow-brown granules	All cells contain pale yellow granules. Occasional blue crystals lying on periphery of cell	15–20 per cent. of cells contain yellow granules.
„ + 1·0 per cent. H_2O_2	Ditto	All cells positive with both yellow- and blue-coloured granules	Ditto, but less intense colour
75 per cent alcohol	Negative except for 1 or 2 cells	All cells coloured yellow. Granules cannot be distinguished	All cells contain faint yellow granules.
Ditto + 0·2 per cent. H_2O_2	All cells contain yellow-brown granules	All cells coloured pale yellow. Granules cannot be distinguished	Negative except for 1 or 2 cells.
„ + 1·0 per cent. H_2O_2	Ditto	Ditto	Ditto.
Solutions each containing benzidine to saturation.			
45 per cent. alcohol	Negative except for 1 or 2 cells	All cells contain yellow-brown granules	All cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	All cells contain yellow-brown granules	Ditto	Ditto.
„ + 1·0 per cent. H_2O_2	All cells contain yellow granules	Ditto, but less intense in colour	All cells contain yellow granules.

All cells referred to in the above table are polymorphonuclear leucocytes.

TABLE II.—*Blood from Dog.*

0·5 per cent. benzidine is contained	Films unfixed, dried in air.	Films fixed in 5 per cent. formalin.	Films fixed in absolute alcohol.
Absolute alcohol	Negative except for 1 or 2 cells	Negative except for 1 or 2 cells	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	Ditto	„ „ „	Ditto.
„ + 1·0 per cent. H_2O_2	„	„ „ „	„
95 per cent. alcohol	Negative except for 1 or 2 cells	All cells contain yellow granules { in large cells colour ++++ / „ small „ „ +++ }	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	Ditto	Ditto { „ large „ „ +++ / „ small „ „ ++ }	Ditto.
„ + 1·0 per cent. H_2O_2	„	„ { „ large „ „ ++ / „ small „ „ + }	„
90 per cent. alcohol	Negative except for 1 or 2 cells	All cells contain yellow granules and cells are surrounded by deep blue acicular crystals	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	Ditto	All cells contain yellow granules less intense in colour and granules present	50 per cent. of cells contain yellow granules.
„ + 1·0 per cent. H_2O_2	„	All cells contain pale granules and deep blue acicular crystals surround the cells	5 per cent. of cells contain yellow granules.
85 per cent. alcohol	Negative except for 1 or 2 cells	All cells negative. Few blue crystals surround the cells	Negative except for 1 or 2 cells.
Ditto + 0·2 per cent. H_2O_2	All cells contain yellow-brown granules	All cells contain pale yellow granules. Occasional blue crystals lying on periphery of cell	15–20 per cent. of cells contain yellow granules.
„ + 1·0 per cent. H_2O_2	Ditto	All cells positive with both yellow- and blue-coloured granules	Ditto, but less intense colour
75 per cent alcohol	Negative except for 1 or 2 cells	All cells coloured yellow. Granules cannot be distinguished	All cells contain faint yellow granules.
Ditto + 0·2 per cent. H_2O_2	All cells contain yellow-brown granules	All cells coloured pale yellow. Granules cannot be distinguished	Negative except for 1 or 2 cells.
„ + 1·0 per cent. H_2O_2	Ditto	Ditto	Ditto.
Solutions each containing benzidine to saturation.			
45 per cent. alcohol	Negative except for 1 or 2 cells	All cells contain yellow-brown granules	All cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	All cells contain yellow-brown granules	Ditto	Ditto.
„ + 1·0 per cent. H_2O_2	All cells contain yellow granules	Ditto, but less intense in colour	All cells contain yellow granules.

All cells referred to in the above table are polymorphonuclear leucocytes.

TABLE II.—*Blood from Dog.*

0·5 per cent. benzidine is contained in each of the following solutions.	Films unfixed, dried in air.	Films fixed in 5 per cent. formalin.	Films fixed in absolute alcohol.
Absolute alcohol	Negative except for 1 or 2 cells	12–15 per cent. of cells contain yellow-brown granules	12–15 per cent. of cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	Ditto	Ditto	Ditto.
„ + 1·0 per cent. H_2O_2	„	„	3 per cent. of cells contain yellow granules.
95 per cent. alcohol	12–15 per cent. of cells contain yellow-brown granules	12–15 per cent. of cells with deep yellow-brown granules	12–15 per cent. of cells with deep yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	Ditto (paler)	12–15 per cent. of cells with yellow-brown granules	All cells contain yellow-brown granules.
„ + 1·0 per cent. H_2O_2	Negative except for 1 or 2 cells	12–15 per cent. of cells with yellow granules	All cells contain deep yellow-brown granules.
90 per cent. alcohol	12–15 per cent. of cells contain yellow-brown granules	All cells contain yellow-brown granules	All cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	12–15 per cent. of cells contain yellow granules	Ditto. Cells are surrounded by blue crystals	All cells contain pale yellow-brown granules.
„ + 1·0 per cent. H_2O_2	12–15 per cent. of cells contain faint yellow granules	Reaction similar to above, but colour more intense	All cells contain yellow granules.
85 per cent. alcohol	All cells contain yellow-brown granules	All cells contain yellow-brown granules.	All cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	50 per cent. of cells contain yellow-brown granules	All cells contain brown granules. Cells appear fissured and surrounded by blue crystals	Ditto.
„ + 1·0 per cent. H_2O_2	All cells negative	Reaction similar, but brown colour more marked	12–15 per cent. of cells contain yellow-brown granules.
75 per cent. alcohol	All cells contain yellow-brown granules	All cells contain brown granules	All cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	10–15 per cent. of cells contain yellow-brown granules	All cells contain yellow-brown granules	12–15 per cent. of cells contain yellow granules.
„ + 1·0 per cent. H_2O_2	All cells negative	12–15 per cent. of cells contain yellow-brown granules	Negative except for 1 or 2 cells.
Solutions each containing benzidine to saturation.			
45 per cent. alcohol	All cells contain brown granules	Negative except for 1 or 2 cells	All cells contain yellow-brown granules.
Ditto + 0·2 per cent. H_2O_2	All cells contain yellow-brown granules	All cells contain yellow-brown granules	Ditto.
„ + 1·0 per cent. H_2O_2	10–15 per cent. of cells (large in size) contain yellow granules	12–15 per cent. of cells contain yellow-brown granules	„

All cells referred to in the above table are polymorphonuclear leucocytes.

When smears treated with benzidine are compared with those obtained by other oxidase and peroxidase reagents, such as the Graham α-naphthol-pyronin and the various modifications of the α-naphthol and paraphenylenediamine, the above-discussed variations are not found. In benzidine, therefore, we possess a microchemical reagent which is more sensitive to changes in the composition of the leucocyte than in these other methods. We were able to compare the blood-films from one case of acute myelogenous leukæmia with normal human blood-cells. In the former there was no appreciable difference in the staining from those of the normal.

In a case of lymphatic leukæmia, under none of the conditions of the experiments indicated in Table I did we obtain staining of the lymphocytes.

Since alcoholic benzidine is a sufficiently sensitive reagent to indicate microchemical differences in myelogenous cells of various animals and under varying conditions, it was thought not unlikely that evidence of the grosser changes known to occur in pus-cells might also be revealed by this reagent. With this object in view, preparations of human pus were studied in a manner similar to that of normal blood except that fixation with absolute alcohol was omitted. Smears were studied both with and without counterstain. With pus, as might be expected, different results depending upon the severity and kind of infection and the amount of change which has taken place in the cells can be obtained. Where cells are very much degenerated and contain many bacteria the whole film is negative. Our most instructive results were obtained with pus taken from three cases of empyema of pleura and a case of suppurative arthritis of the knee-joint. As the results obtained with all of these samples of pus resemble one another closely only one will be described. The details of this are included in Table III.

All four samples of pus were removed by aspiration and studied as soon after removal as possible. When kept in the refrigerator fairly concordant results could be obtained for two or three days, but the preparations slowly degenerated and the positive reaction in the granules gradually changed to a negative. If the sample is allowed to stand at room temperature for three or four days it is converted into a negatively reacting specimen. Not only does the pus in the test-tube gradually lose its affinity for the benzidine, but the unstained as well as the stained films react negatively with the lapse of time. In the above four cases with the various concentrations of alcohol, a series of colours ranging from deep brown through yellow and yellow-green to blue were observed in the granules following immersion in benzidine solution. The blue colour is very sharply defined and is most commonly found with concentrations of alcohol of 85 and 90 per cent., though it may even be very distinct in the solutions made up with alcohols of 95 and 75 per cent. In the two latter concentrations, cells containing mixtures of yellow and blue granules are common, but many cells are filled with only the blue or the yellow granules. This blue colour found in pus is identical with that developing in granules of

normal cells of both human and dog's blood to which acid has been added, and indeed if a trace of acid from the cleaning solution accidentally remains on the slide on which the pus smear is spread the blue colour in the granules appears immediately. The appearance of this blue colour is very much like that which is figured and described by Graham ([5]) in cells following treatment with weak acids. The four samples of pus studied were neutral to litmus. Formalin fixation prevents the formation of this blue colour, which appears to be much less resistant than the yellow. Variations in the intensity of the yellow colour

TABLE III.—*Pus from Empyema of Pleural Cavity.*

0·5 per cent. benzidine is contained in each of the following solutions.	Films unfixed, dried in air.	Films fixed in 5 per cent. formalin.
Absolute alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	Ditto	Negative except for bluish colour in nuclei.
„ + 1·0 per cent. H_2O_2	„	Negative.
95 per cent. alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	Cells contain both yellow and blue granules	Ditto.
„ + 1·0 per cent. H_2O_2	All cells contain blue granules	Negative except for pale-blue colour in nuclei developing immediately on immersion and lasting only a few minutes.
90 per cent. alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	All cells contain blue granules	All cells contain brown granules.
„ + 1·0 per cent. H_2O_2	Ditto	All cells contain deep brown granules.
85 per cent. alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	All cells contain blue granules	All cells coloured yellow. Granules not distinct.
„ + 1·0 per cent. H_2O_2	Ditto	All cells coloured yellow. Occasional brown granules distinct, but reaction tending to become diffuse.
75 per cent. alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	Cells contain both yellow and blue granules	All cells contain yellow-brown granules. Transient blueing of nuclei.
„ + 1·0 per cent. H_2O_2	Most of the cells contain blue granules, but a few show both yellow and blue granules	Ditto.
Solutions each containing benzidine to saturation.		
45 per cent. alcohol	Negative	Negative.
Ditto + 0·2 per cent. H_2O_2	All cells contain brown granules	All cells contain brown granules. Transient blueing of nuclei.
„ + 1·0 per cent. H_2O_2	All cells contain deep brown granules	Ditto.

from a very faint yellow to a dark brown occur. In formalin preparations the maximum brown colour appeared with 45 per cent. alcohol *plus* 0·2 per cent. hydrogen peroxide.

After formalin fixation, with the solutions made up with 90 and 95 per cent. alcohol and containing hydrogen peroxide, a faint blue colour develops in the nuclei immediately upon immersion. This colour is very evanescent, and disappears if the film is allowed to remain in the reagent for more than one or two minutes. When the preparations are counterstained it is completely obscured. If absolute instead of 90 or 95 per cent. alcohol is used as a solvent the blue colour is more pronounced, but it is then only observed when the hydrogen peroxide is present in a concentration of 0·2 per cent.

Pus from abscesses and discharging sinuses invariably gives a negative reaction, in which case the cells show considerable degeneration.

DISCUSSION.

A glance over the results recorded in Tables I and II will suffice to convince one that widely differing effects may be obtained with benzidine as a microchemical reagent. By varying slightly the alcohol or the hydrogen peroxide of the reagent the same film may be transformed from a positive to a negative. Films from different types of animals do not always give identical pictures when immersed in the same solution.

Because the benzidine reagent responds by colour changes to such slight variations in its composition, its usefulness as a means of differentiation of cells of myelogeneous origin is lessened unless these variations are controlled. It does, however, offer possibilities of being adapted as a microchemical method for studying minor differences among these cells.

That minor differences exist between the red blood-cells of various animals has already been well established by the separation of different crystalline forms of hæmoglobin and by the finer serological technique. Methods for demonstrating the existence of analogous differences in leucocytes are at present not available, but that similar variations do obtain is indicated by results obtained by alcoholic solutions of benzidine. While the latter lack the precision and delicacy which marks the serological technique, that further modifications may show still finer differences in the composition of these white blood-cells is suggested by Graham (7) in his work on basophiles. As has been already indicated, the addition of hydrogen peroxide in minute quantities so modifies the reaction of the reagent that it will differentiate the human cells of myelogenous origin from those of many of the lower animals.

While it is probable that these variations are due in large part to actual differences in the cellular composition, it is not to be overlooked that part of the variation may also be due to physico-chemical changes induced by slight differences in the media surrounding the cells. Thus, for example, by exposure of blood-films to traces of acid, colour formation in cell granules analogous to

that found in samples of pus may be developed. While the degree of acidity required to give this reaction is certainly never reached in normal blood *in vivo*, the quantitative as well as qualitative differences in the salt content of blood-plasma of various organisms will exert a similar (or opposite) action, though in a smaller degree.

That variations in the hydrogen-ion content of the environment operate *in vivo* as a modifying factor is clearly demonstrated in colour results obtained in samples of pus aspirated from cavities in the body. Whether the colour-reaction depends, as has been postulated by various investigators, upon the presence within the cell of the hypothetical substances, the so-called oxidases and peroxidases, which are capable of directly transferring oxygen from the cell constituents to certain dyes, seems to be very doubtful. It would seem that, rather than looking upon these substances as chemical entities, the phenomenon of intracellular colour development might be more readily explained as being due to physico-chemical changes in tissues—a view which we have already discussed in two previous papers (8), (9).

Since all of the colours developing under the experimental conditions described in this paper are due to oxidation of the benzidine, and oxidation always results directly or indirectly from the acquisition by the oxidised body of positive electrical charge, the reaction is due fundamentally to a transfer of this charge in part or whole from the cellular constituents to the dissociated particles of benzidine which they have taken up. That the inclusion of this base by the cell is an adsorption process is strongly suggested by the observation that the benzidine taken up by the cells lies on the periphery of the granules instead of being incorporated within them—a fact which strengthens the assumption of the physico-chemical nature of oxidases and peroxidases.

SUMMARY.

(1) The Graham benzidine peroxidase reagent, consisting of a 0·5 per cent. solution of benzidine in 75 per cent. alcohol *plus* 0·2 per cent. hydrogen peroxide, produces a yellow-brown colour in the granules of myelogenous cells in human blood and is diagnostic for man.

(2) The polymorphonuclear leucocytes of blood of lower animals studied differ from those of human blood in their reaction to the Graham reagent, in that only a small percentage of them develop the characteristic yellow colour. These positively reacting cells are probably eosinophilic.

(3) In a 0·5 per cent. solution of benzidine in 75 per cent. alcohol free from hydrogen peroxide practically all of the myelogenous cells of the guinea-pig, dog, white mouse, white rat and rabbit give the characteristic coloured granular reaction. Addition of hydrogen peroxide to this solution tends to inhibit the development of the yellow colour, which disappears from all except one or two cells when the concentration of the peroxide is increased to 1 per cent.

(4) By varying the concentrations of the alcohols in the Graham reagent a series of colours ranging from deep brown through the yellows and the yellow-greens to blue may be obtained in granules of human pus aspirated from empyemic cavities. As these samples of pus age, the colour reactions are lost. Pus in which the leucocytes become more degenerated give a negative reaction.

(5) The blue reaction of pus-cells may be duplicated by exposing blood-films to weak concentrations of acid, previous to treatment with benzidine solutions.

REFERENCES.

1. Schultze, W. H.—*München. med. Wochenschr.*, 1909, **56**, 167.
2. Fischel, R.—*Wien. klin. Wochenschr.*, 1910, **23**, 836, 1557.
3. Fiessinger, N., and Roudowska, L.—*Arch. de méd. expér. et d'anat. path.*, 1912, **24**, 585.
4. Loele, W.—*Ergebn. d. allg. Pathol. u. pathol. Anat.*, 2te Abt., 1912, Jahrg. 16, 776; *Folia hæmatol.* 1te Abt., 1912, **13**, 331; 1te Abt., 1912, **14**, 26.
5. Graham, G. S.—*J. Med. Res.*, 1918, **39**, 15.
6. Graham, G. S.—*Ibid.*, 1916–17, **35**, 231.
7. Graham, G. S.—*J. Exper. Med.*, 1920, **31**, 209.
8. Menten, M. L.—*J. Med. Res.*, 1919, **40**, 433.
9. Menten, M. L.—To appear in *J. Cancer Res.*, 1921, **6**.

RESEARCHES ON DYSENTERY.

F. W. TWORT, M.R.C.S., L.R.C.P.

The Brown Institution, University of London.

Received for publication July 16th, 1920.

DURING 1913, 1914, and 1915 I conducted a series of experiments for the Local Government Board on the nature of Ultramicroscopic Viruses, and the main results obtained were published in the *Lancet*, December 4th, 1915. In these experiments it was shown that vaccinia often contained micrococci which failed to grow on account of the presence of an unknown material which dissolved the cocci before the colonies had grown sufficiently to be visible. It was also shown that this dissolving material passed through a porcelain filter, that it was able to attack and dissolve into granules a pure culture of the micrococcus, and that from fresh cultures, the growth of which had been made transparent, it could be carried through an indefinite number of generations to fresh cultures of the micrococcus, although no visible growth of the dissolving material by itself was obtained. The activity of the material was retained for six months, but it was destroyed when heated to 60°C. for one hour. The same general results were obtained with a member of the coli-typhoid group of bacilli, and it was suggested that the difficulty often experienced in isolating certain known pathogenic micro-organisms might be due to the same cause. In cases of infantile diarrhœa similar results were obtained with some rather large bacilli, thought at the time not to belong to the dysentery-typhoid-coli group but since believed to be a variety of this group. In the paper referred to, the relation between the dissolving material and the bacterium was discussed, as was also the possibility of the dissolving material being an ultramicroscopic virus. It was also suggested that similar results would be obtained in cases of dysentery and allied conditions to be met with in the Dardanelles.

Some experiments by Dr. F. d'Herelle, presented by Prof. Roux, and published in the *Comptes Rendus, Academie des Sciences* (No. 11, 10th September, 1917), show results which confirm those given above. Dr. d'Herelle has worked chiefly on dysentery, and has obtained a filter-passing substance which dissolves up the Shiga bacillus. He states that the dissolving material can be acclimatised to act on other bacilli, and from the experiment that shows

the dissolving action to start from distinct spots or areas he concludes that the substance is an ultra-microscopic virus. As I have already stated, I do not think that this point has been proved in view of the experiment in which I obtained an *apparent* spontaneous start of the dissolving action in cultures of the coccus which appeared to have remained free from the dissolving material for many generations previously. However, although this is an important point it does not affect the main experimental facts.

In the original experiments conducted at the Brown Institution some additional observations were made besides those which have already been mentioned. These were not published at the time as their exact significance had not been fully determined. It is a well-known fact that various species of the dysentery-typhoid-coli group when apparently in pure growth produce forms that are considerably longer than the typical short forms, and variations in the size and shape of individual bacilli in a pure culture have often been described. Recently Hort and others have carried out a considerable number of experiments with the object of throwing light on the significance of these different forms.

In certain experiments dealing with the dissolving material I observed these long forms in greater number than usual, and it was thought that they might in some way be connected with the dissolving material which had also been found associated with this group. On the other hand there was the obvious possibility that they might be distinct but symbiotic bacilli, or again they might represent a stage in the life history of the bacillus. It was also thought possible that they might be special forms with special work to do like bees in a swarm, and might prove to be of importance in connection with the pathogenicity of the cultures and the production of immunity in the host, or they might represent nothing more than a mutation.

Here I may mention that in 1914 I advised that dysentery would probably occur in the Army, and suggested that the whole problem of dealing with this condition should be worked out in advance. Unfortunately the War Office did not accept the scheme of research which was submitted, so I had no opportunity of testing these points, which would have formed part of the general investigation.

Attempts, however, were being made at the Brown Institution to isolate the large forms of bacilli, when at the end of 1915 the work was interrupted, and I was asked by the War Office to organise and take out the Base Laboratory to Salonica. Soon after arriving I again advised that a systematic investigation should be carried out on dysentery with the object of obtaining an effective vaccine or serum against this condition, but once more I failed to obtain official approval, although I constantly gave expression to my views. As I had expected, the outbreak of dysentery, which at first was almost entirely bacillary in nature, arrived in due course, and it was not long before we were inundated with routine work. At the same time most of the staff became more or less ill and, while we were able to confirm the results already mentioned, it was impossible for me to carry the investigation much further.

The absence of official approval of my proposals no doubt was partly due to the fact that we came into strong conflict with the "Medical Advisory Committee" in Egypt, who concluded that the majority of cases of dysentery in the East were due to amœbic infection, and had criticised our diagnosis that the infection in Salonika was bacillary in nature. Lieut.-Col.

Buchanan, a member of the Committee, has since admitted in his presidential address before the Epidemiological Section of the Royal Society of Medicine, December 7th, 1917, that he knew "very little of dysentery on the practical side," and that the Committee had fallen into the error of mistaking body-cells in the discharge for parasitic amœbæ in case after case of dysentery; but this admission was made after we had returned from Salonika and it was therefore too late to undo the damage done by their first reports. However, the work was continued on my return, and that portion of the investigation on dysentery which is given in this paper would have been published in 1918 had I not included a criticism of the War Office and the Medical Advisory Committee for failing to take what I considered to be the necessary precautions against dysentery and for the erroneous diagnosis of cases in the East. As it was I was unable to get my paper published. Moreover, about the middle of 1918 I was ordered to rejoin the R.A.M.C., and it then became impossible for me to carry the matter further.

On returning from Salonika, as I have said, some additional experiments were carried out and some interesting results obtained. In the first place the long forms, after some difficulty, were isolated from ordinary "pure cultures" of various types of dysentery, from typhoid, and from certain coliform bacilli.

The medium used consisted of beef extract, 1 lb. to 2 litres of distilled water. To this, after filtration, were added 1 per cent. peptone, and $\frac{1}{2}$ per cent. of sodium chloride. A solid medium was obtained in the usual manner by adding 2 per cent. of thoroughly washed agar. This was cleared with the yolk and white of egg, and to the clear agar were added 0·1 per cent. sodium bicarbonate, 0·5 per cent. of litmus solution and 0·5 per cent. maltose. The medium was now tubed and autoclaved.

The strains of bacilli to be tested were plated and replated several times to ensure purity. This was done by inoculating the condensation water of sloped tubes in a series of dilutions, and flooding this over the surface. Colonies on the last plates were then taken as pure cultures and placed on tubes of the medium described above. Observation demonstrated that these cultures contained a few units which were much larger and thicker than the normal bacilli, and that they were more numerous in the very young cultures than in older ones. Repeated subculturing from very young cultures caused a relatively large number of the big forms to grow, and after plating out such cultures on the surface of sloped tubes of the same medium, colonies containing a large number of the big forms were obtained. The cultures thus isolated agreed fairly closely with the original cultures in fermentation reactions and in agglutination tests, but the growths on solid media presented a much more spreading and wavy character. Some of the colonies when young appeared to be associated with a slowly acting dissolving material, similar to that described above, and subcultures from such colonies often failed to grow.

In cultures of the "large forms," the bacilli grew with considerable variation in length, thickness and shape, and a further series of experiments was made with a typical strain of the Shiga type of dysentery in order to separate if possible the different large forms, the same medium being used. A culture of the big forms which was isolated from this Shiga was grown under a nearly complete anaërobic condition and the growth was then plated out

aërobically on sloped tubes of the maltose medium. Two distinct types of colonies were obtained. One, which will be referred to in future as type A, grew as large mottled colonies. Stained films and hanging drop preparations of this type showed many long, thin, wavy threads, sometimes, when observed under a $\frac{1}{12}$ oil immersion lens, extending half way across the field of the microscope. These threads are often twisted like a spirochæte (Fig 2). They vary considerably in length—some, possibly very young members, being quite short—whilst a few granules are sometimes present. The second type of colony, which will be called type B, grew at first as small grey colonies, but later spread considerably and became markedly mottled. Films and hanging drop preparations from this type present some very interesting morphological characters. The units, as a rule, are long like the first-mentioned thread forms, but thicker (Figs 3 and 4), and are often markedly swollen, either in the middle or at one end (Fig 4). The large swollen ends are sometimes split open, and are seen to have set free numerous granules; moreover a large number of these granules can be found free in all film preparations (Fig 3). How far these may be connected with the dissolving material already described has not yet been determined. Some units of a culture are granular and almost globular in shape, and these may attain the size of a yeast-cell or may be not much larger than a coccus.

On replating type B, a third form, or type C, was obtained. Colonies of this type are usually smaller, and are more waxy in appearance and less mottled. Film preparations show thick bacilli which grow considerably shorter than type B, but present the same swollen ends and the same granules as seen in that type (Figs. 5 and 6). The round granular forms are present in large numbers, especially in cultures that are several days old, where most of the units present a globular shape. In older cultures the protoplasm collects as a semilunar mass round the circumference of each unit; at least there is very little stain taken up by the rest of the cell (Fig. 7). In young cultures short, swollen, branched forms are often seen; these, as a rule, are roughly triangular in shape—that is, they present three short tapering outgrowths from a central granular protoplasmic mass. Longer branched forms are also to be seen (Fig. 6).

Some of the characters of the special forms A, B, and C are best observed when they are grown at between 25° C. and 30° C., but this is not essential. It may be noted also that although these forms were isolated on a maltose medium, subcultures made on to ordinary peptone-salt-broth agar show the characters equally well.

It is important to note that the large forms can always be isolated from the short or normal form, provided suitable means are employed, but it has not yet been ascertained for certain that the short forms can be recovered from any of the long or special forms, although cultures of these types may contain very short bacilli. Types A, B, and C show a tendency to grow these

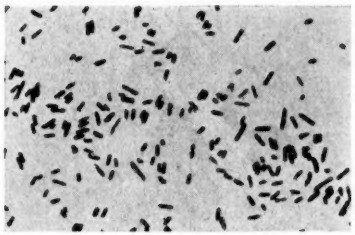


FIG. 1.—Normal type, 24 hrs. at 37° C.

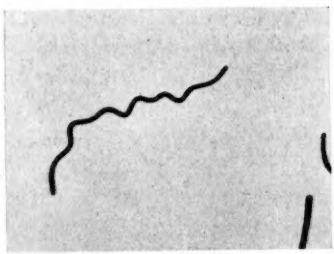


FIG. 2.—Type A, 5 hrs. at 37° C.

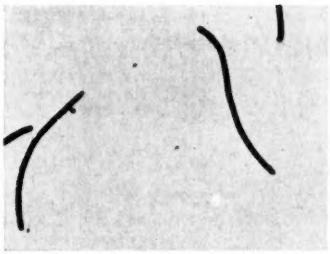


FIG. 3.—Type B, 5 hrs. at 37° C.

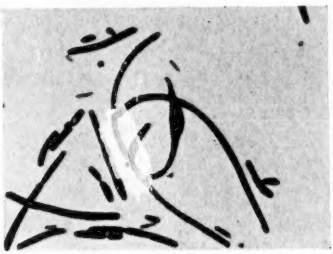


FIG. 4.—Type B, 24 hrs. at 25° C.

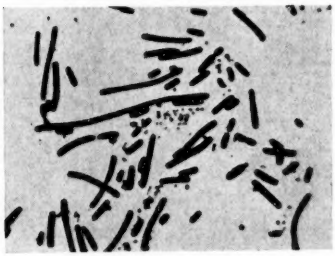


FIG. 5.—Type C, 5 hrs. at 37° C.

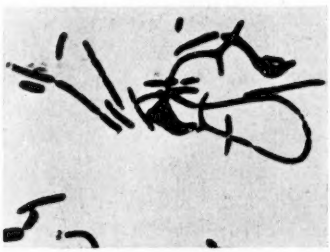


FIG. 6.—Type C, 24 hrs. at 25° C.

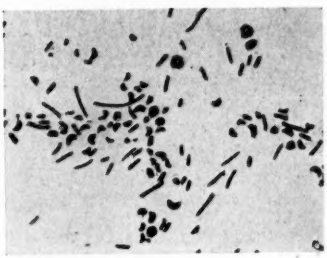


FIG. 7.—Type C, 3 days at 25° C.

CULTURES OF SHIGA'S DYSENTERY BACILLUS ON PEPTONE-SALT-BEEF-BROTH AGAR.

Twort. Researches on Dysentery.

forms mixed after a number of subcultures have been made, and this is particularly so with type A, which occasionally produces baccilli which, when isolated in plate cultures, show the characters of type B. Types B and C show greater variation in the shape of their units when first isolated from a normal culture than after repeated subculturing of the types.

In the case of certain of the coliform bacilli, the morphological differences between the different forms are much less marked than with the dysentery bacilli.

The exact relation of the "dissolving material" and the "granules" to the bacilli has not yet been determined, but it may be noted that they have never been grown apart from cultures of the bacilli.

Colonies of type C, and to a less extent those of type B, are often sticky and viscid like mucus, and this is usually more marked when they are grown between 25° C. and 30° C. In fermentation tests types A, B and C do not vary much from the standard small type, although on litmus-maltose-agar slopes type C produces marked acidity, type B fair acidity, while with type A there is often no acidity except near the condensation water. Types A, B and C agglutinate with dysentery agglutinating serum to a higher titre than does the normal type, but as spontaneous agglutination is very difficult to avoid with the special types, it is impossible to give detailed comparative tests. It is, however, quite clear from the experimental results that the special forms are not, as was at one time thought possible, distinct symbiotic bacilli, and the additional experiments carried out with typhoid and with coliform bacilli and their large forms confirm this view.

It may also be noted that the special forms do not appear to represent a type of degeneration, since they are best obtained by using good media and by keeping the cultures under good conditions.

It is, of course, possible that bacteria, like the spirogyra and other low forms of life, may not only multiply asexually, but also sexually. It may, in fact, be that a sexual form of multiplication is essential to the maintenance of a vigorous life after a certain number of asexual divisions, and it was thought that the special forms described above might represent the sexual units. Experiments carried out to test this were all negative. Mixtures of the special forms, after repeated subculture in broth, failed to give colonies of normal bacilli. If sexual forms do exist they must be present in the normal culture, since it is from this that the special forms are obtained. On the other hand, the fact that the special forms, when subcultured on ordinary agar, as they have been for several years now, do not reproduce normal cultures in rotation, is a strong argument against their being stages in a true life-cycle. It may be that they are nothing more than mutations, but if so it is a little strange that although a few can always be found in young cultures of dysentery bacilli, yet they never appear to survive under normal conditions when a normal culture is grown on ordinary laboratory media: the

normal forms, although apparently producing the special forms, nevertheless soon outgrow them. It may be that the "dissolving material" plays a part in this process.

Another possible explanation appears to be that the large forms are specialised units which are produced by the normal bacilli for some special purpose. They may assist in producing assimilable food or protective substances for the normal bacilli whose chief function is to reproduce. If such is the case it might explain why the special forms do not normally survive in cultures made on laboratory media. It might also explain certain points regarding successful infection, incubation period, the termination of infection, and the production of toxins, antitoxins, etc. In this connection it is interesting to note that in specimens from the human body of such material as fæces and urine, long forms of bacilli are often present in considerable number, and this is especially so in specimens from the upper part of the intestinal tract in cases of infantile diarrhœa and vomiting, and in specimens of urine in cases of cystitis; and it does not seem at all impossible that these forms may play an important *rôle* in producing the toxic effects in such conditions. This is a point that should be investigated further, for it might lead to the production of more efficient vaccines and possibly also of antitoxins.

Some experiments were carried out to test the toxicity of the special forms for animals. In most cases mice and rabbits were used, and the results appeared to show a greater toxicity of these forms than the normal cultures, but they were not sufficiently marked to draw any definite conclusions. It is, however, worthy of note that the special forms appear to lose their toxicity rapidly when they are isolated from the small forms, and it may be that their power of producing toxic substances is marked only in those units which are produced directly from the small forms. The fact that in a normal culture the special forms appear to be produced in this manner and then die out without showing any evidence of multiplication by simple division, gives support to this suggestion. If effective toxin-producing units must be produced directly from the small forms, it is possible that the use of the dissolving material is to destroy them after they are no longer of any use to the bacterial community, or the dissolving up may be a means of setting free the enclosed toxic substances. In the case of the micrococcus which was isolated from vaccinia, the breaking down of the organisms may extend to the whole culture, and in a few hours no living units may be left; but conditions of cultivation outside the body are very artificial, and it seems improbable that in the infected body this process would extend further than was beneficial to the life of the bacterial community.

If then, as seems possible, the function of some of the special forms of dysentery bacilli is to produce toxins or other substances for the protection of the bacterial community, then any conditions which might lead to the production of these forms would assist the normal bacilli to survive and

multiply when they gained entrance to the human body. In this connection my wife, then Miss Banister, devised and carried out a series of experiments to test for the production of large forms in media that contained a high percentage of sodium chloride, the idea being that the climatic conditions which favour an outbreak of dysentery would also produce a higher concentration of salt in their environment. In these experiments the maltose medium described above was used as a basis, and 1 per cent. of sodium chloride was added in place of 0·5 per cent. A pure growth of normal Shiga bacillus was inoculated on this medium, and repeated subcultures made on to fresh tubes. In these cultures large forms were produced as in the previous experiments, but in addition a few extremely large branching threads were observed. Their length was considerably more than the diameter of the microscopic field, and they appeared more like the mycelium of a mould than anything else. These threads contained granular protoplasm, and as a rule remained but faintly stained. The experiments were repeated several times with the same results, but all attempts to isolate these forms failed so that it was impossible at the time to investigate them further, and their significance could not therefore be determined. It may be noted, however, that great care was taken to eliminate contaminations, and I think this possibility can be excluded.

More recently, with the assistance of my wife, experiments have been carried out for the Medical Research Council on influenza, and somewhat similar results have been obtained. We hope to publish a paper on these investigations shortly.

SUMMARY.

Three types of special forms can be isolated from dysentery and allied bacilli. Type A shows moderately thick but long bacilli, which may twist like a spirochæte. Type B shows long thick rods; some swollen in the middle or at one end, and these may break open and set free granules. The bacilli are associated with round forms and many free granules. Type C bacilli are like type B, but shorter. There are many granules and round forms, also thick branching units. In older cultures most of the units are globular, and the protoplasm may collect as a semi-lunar mass round the circumference. A filter-passing dissolving material may be present in cultures of dysentery bacilli, etc., which breaks down the bacilli into granules. The special types A, B and C are produced by normal bacilli, but some smaller units are always present. The special forms are probably not stages in a true life-cycle, as the normal type is not produced in rotation, and it is doubtful if they are mutations. Special types may be produced for performing special work, and types in "pure culture" may be likened to bees in a swarm. They are possibly of importance in connection with incubation period, successful infection, and production of immunity. Sexual multiplication is not proved, but may exist in normal cultures.

THE RESPIRATORY EXCHANGE OF SURVIVING MOUSE TISSUES, NORMAL AND NEOPLASTIC.

B. R. G. RUSSELL AND W. H. WOGLOM.

From the Laboratories of the Imperial Cancer Research Fund, London.

Received for publication September 1st, 1920.

IN a previous number of this Journal one of us in collaboration with W. E. Gye (1) published an account of experiments bearing upon the oxygen consumption of normal and cancerous mouse tissues *in vitro*. The reasons for carrying out the experiments *in vitro* instead of by the preferable method of *in vivo* were detailed there and need not be entered into again. Shortly stated, the oxygen consumption was ascertained in those experiments by measuring the amount of oxygen which disappeared from a known amount of defibrinated rabbit's blood, after incubation with normal or cancerous mouse tissues. We also attempted to estimate the carbon dioxide of the blood, but the measure of success which attended the estimation of oxygen was not repeated in the case of the other gas, for the figures obtained were so erratic as to be quite unusable. It was impossible to strike any average which would give a reasonable respiratory quotient, and with it information as to the probable food substances mainly consumed.

It may be recalled that the respiratory quotient expresses the ratio of the carbon dioxide excreted to the oxygen absorbed. In the case of carbohydrates these two volumes are equal and the quotient is unity. When fats are metabolised there is relatively less carbon dioxide given off, and a quotient of 0·71 is found. The quotient for proteins varies slightly, but is usually about 0·81. Sometimes a respiratory quotient exceeding unity is observed, as in the case of some animals after a meal rich in carbohydrate. This has usually been interpreted as meaning a conversion of part of the carbohydrate into fat. A quotient below 0·7 generally means incomplete oxidation with the formation of intermediate metabolic products. If the respiratory quotient be known an idea is obtained of the respective food-stuffs being burnt up, and for this reason it seemed desirable to ascertain the quotient for a number of different tissues and tumours.

In the present communication an account will be given of the respiratory exchange as ascertained by a different method, which, although in some ways inferior to the previous method, has nevertheless supplied us with results which in their consistency have surpassed what we had reason to expect.

The method used was to measure the gaseous exchange of a tissue emulsion exposed at blood heat in a gas chamber. The difficulty of providing a suitable apparatus was overcome by using an ordinary Barcroft apparatus as made for the analysis of gases in 1 c.c. of blood. Krogh (2) has pointed out that this apparatus can be used for respiratory estimations on small animals, and has devised a modified form which is an extremely sensitive respirometer, and can measure the oxygen absorption of a single insect egg weighing 2 mgrm. For mouse tissues with a high oxygen consumption, such as kidney, the ordinary Barcroft apparatus gives quite good results, but for most tumour tissues which have a lower gaseous interchange we have found it necessary to have an apparatus made* which was more sensitive, and which at the same time allowed more tissue to be introduced. The apparatus has been calibrated for the varying conditions during the experiment by the method described by Hoffmann (3).

When an experiment is to be carried out, a large water-bath provided with an adequate mechanically driven stirrer is heated up to 38° and kept steadily at this temperature. The respirometer is then hung on the bath with two bulbs completely immersed. This preliminary warming shortens the period necessary to attain equilibrium later. The animal is killed, and the tissue to be used is rapidly excised and minced with curved scissors until an apparently homogeneous emulsion is obtained. Part of the emulsion is spread evenly on a rectangular slip of ground-glass, the amount being ascertained from the difference in weight of glass before and after. Weighing has been done on a Collot balance, which reduces the time taken and evaporation to a minimum. The lower portion of the slip of glass must be kept free of tissue, otherwise there will be contact with the alkali which is added later. The glass slip with the tissue is placed in one bulb of the respirometer, with a corresponding glass slip in the other, and both bulbs are filled with expired air and replaced on the apparatus, which is returned to the water-bath. The time elapsing between the death of the animal and the commencement of incubation has varied between ten and fifteen minutes.

Expired air has been chosen because it is relatively constant in its composition, is saturated with water vapour, and contains carbonic acid at a tension approximating to that usually experienced by the tissue. Human expired air has of course been used, but it is probable that mouse expired air does not vary very widely from it in its composition. It was hoped that in using expired air, equilibrium, especially of carbonic acid exchange, would be sooner established, and the results seem to harmonise with the view.

After ten minutes, during which the apparatus is warming up, the taps are turned off and the position of the manometer menisci recorded. Observations are made usually at ten-minute intervals for forty minutes, the figure for the first ten minutes being rejected, as it is usually about fifteen minutes after

* The apparatus has been made for us by Messrs. Orme & Co., 148, High Holborn, W.C. 1.

incubation begins before a steady rate of gaseous exchange is established. Any absorption—that is, a contraction of volume on the side of the apparatus containing the tissue—observed during these forty minutes will represent a surplus of oxygen consumed over the carbonic acid produced, whilst an expansion will represent a surplus of carbonic acid. If no movement be noted it is obvious that the oxygen and carbon dioxide exactly balance, as they do in the case of a pure carbohydrate metabolism with a respiratory quotient of unity. To ascertain the total amount of oxygen being consumed, 1 c.c. of warm 6 per cent. KOH is added to each bulb to absorb the carbon dioxide, and after a suitable interval of ten or fifteen minutes the excursion of the manometer is again read at ten-minute intervals. If the gaseous exchange is constant over the two periods observed a respiratory quotient can be established. The disposition of the apparatus did not allow the maintenance of an atmosphere of uniform composition in the two periods, but as the conditions were maintained in the same way in successive experiments comparative results could be obtained. The duration of the period of observation has been largely determined by the circumstance that we are dealing with cells which are beginning to die, and that the result must be obtained before they have gone too far on their way. In general the displacement of the manometer has been fairly uniform over successive periods of ten minutes, and the following protocol is taken as an example of what may be expected to occur.

1/7/20.—Mouse bearing a transplanted tumour of carcinoma 155 was killed at 2.5 p.m.; the tumour was rapidly excised, and after being freed from all apparent necrotic areas was emulsified with curved scissors. Of this emulsion 286 mgrm. were spread on a glass slip over a surface of 800 square mm., placed in the respiratory chamber, and the respirometer returned to the water-bath at 2.15 p.m. At 2.24 p.m. both chambers were shut off from the air, and the recording of the manometer changes commenced.

Absorption.

p.m.	mm.	p.m.	mm.
2.24	0	3.12	2
2.30	1	3.22	9·75
2.40	3·75	3.32	17·5
2.50	6	3.42	24·5
3	8		

After addition of 1 c.c. 6 per cent. KOH to each bulb, the changes recorded in the second column were obtained: the 7 mm. absorption occurring between 2.30 and 3 equalled 13·33 c.mm. at 0° C. and 760 mm. Hg.; the 22·5 mm. absorption between 3.12 and 3.42 equalled 41·39 c.mm.

$$\frac{41{\cdot}39 - 13{\cdot}33}{41{\cdot}39} = \frac{CO_2}{O_2} = \text{respiratory quotient } 0{\cdot}678.$$

The amount of oxygen consumed was equivalent to 4·8 c.c. per kilogramme minute.

The value of the respiratory quotient thus established will also depend on the evenness of the respiratory exchange during the two periods. This has been controlled in a separate apparatus, where part of the tumour emulsion was incubated over alkali from the start, and the following absorption noted at ten-minute intervals. Starting at zero the successive readings were: 2·25, 5·5, 8, 10·25, 12·25, 14·5, 16·75, 19, 21. The successive increments are sufficiently close to warrant the assumption that there is no appreciable alteration in the character of the respiratory exchange during the period of observation.

The first mouse-tissue to be tested was minced kidney, as this has a very active metabolism, and the results of six observations on the kidneys of different mice are summarised in Table I.

TABLE I.—*Kidney.*

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·893	20·0	61	230
0·884	18·2	93	,,
0·875	12·7	124	,,
0·880	19·2	85	,,
0·884	22·4	76	,,
0·883	15·0	102	,,

Average respiratory quotient 0·883, ± 0·002.

The mean error of the series does not affect the second decimal place, and the respiratory quotient of surviving mouse kidney may be taken as having a mean value of 0·88. The oxygen consumption per kilogramme minute has fluctuated rather widely, but the higher values obtained agree with the average, 21·3 c.c., found for kidney suspended in rabbit blood. In spreading an emulsion of kidney substance on a glass slide it is not always possible to maintain exact uniformity, and the actual quantity used in covering a given surface has varied considerably, as will be seen from the figures in column 3. Comparison of column 2 with column 3 shows an inverse proportionality: the more tissue used above a given optimum the lower the rate of oxygen consumption. As different tissues vary in the facility with which they can be minced and spread, the method is not a good one for measuring comparative oxygen consumptions, although it gives comparative respiratory quotients. The figures for six experiments on liver-tissue are given in Table II (page 248).

The mean error of the series is here ten times as great as that with kidney, and the quotient is appreciably lower. Liver-tissue has been found to be much more labile than kidney, for where the latter will consume oxygen at

an undiminished rate for two hours at least, the liver-tissue will show a falling off much sooner. For this reason we suspect that the respiratory quotient struck for liver is a little too low.

TABLE II.—*Mouse Liver.*

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·868	7·6	82	230
0·826	5·8	80	,,
0·736	7·0	126	460
0·759	7·8	156	,,
0·804	12·5	119	,,
0·887	11·1	154	,,

Average respiratory quotient 0·813, ± 0·024.

In some cases, but not in all, a portion of the liver emulsion was fixed and stained for glycogen. The left-hand portion of Plate-fig. 1, shows the abundance of fine granules of glycogen before incubation, whilst the right-hand portion shows the diminution after one and a half hours' incubation. The renal parenchyma of the mouse does not usually show any glycogen, although its higher respiratory quotient means a more extensive use of carbohydrate during the survival period tested.

Because most mouse tumours are, after all, progressively growing mammary epithelium, we give in Table III figures for the mammæ of five pregnant mice, killed during the last week of gestation. Histological examination of part of the organ showed in all cases a considerable degree of epithelial growth with reduction of the fatty embedding tissue.

TABLE III.—*Mamma.*

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.	Date of pregnancy.
1·000	12·2	136	480	16th day.
0·935	6·7	106	,,	?
0·920	8·5	212	800	18th day.
0·853	10·0	129	480	19th day.
0·861	9·4	158	800	20th day.

Average quotient 0·914, ± 0·027.

This is the highest quotient yet found for normal tissues, and if the individual figures are examined, it will be noted that there is a tendency for it to fall as the end of gestation is approached.

The submaxillary salivary gland, a large readily excisable organ in the mouse, was also examined, and the figures are given in Table IV.

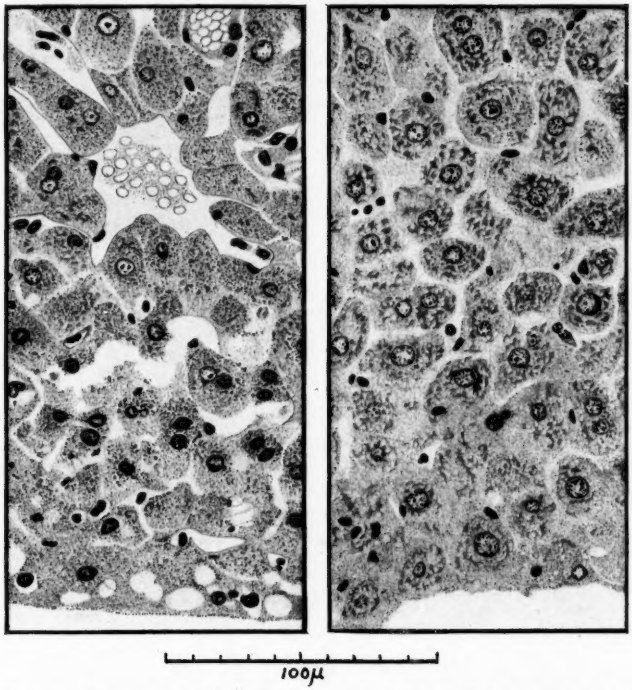


FIG. 1.—Liver stained for glycogen. Left hand: before incubation. Right hand: after 2½ hours' incubation. × 410.

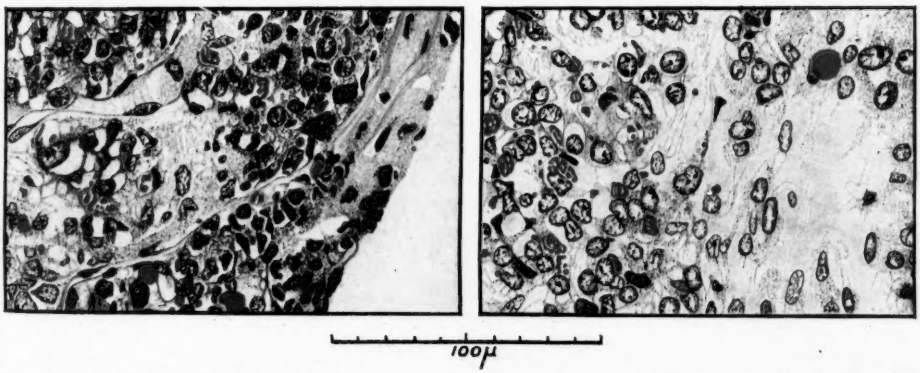


FIG. 2.—Carcinoma 113 stained for glycogen. Left hand: before incubation. Right hand: after 1½ hours' incubation. × 410.

TABLE IV.—*Submaxillary Salivary Gland.*

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·901	9·3	70	240
0·739	10·8	112	480
0·845	7·0	134	,,
0·829	10·5	134	,,
0·832	12·9	201	800
0·901	8·3	174	600

Average respiratory quotient 0·841, ± 0·024.

Before discussing the results obtained with these four tissues, the findings on six transplanted mouse-tumours will be given. Five of these were mammary carcinomata, and one, 37 S., a spindle-celled sarcoma. The carcinomata present great differences in their speed of growth, the fastest being tumour 206, followed by 63 and 113, whilst the two strains 72 and 155 grow very slowly. The two latter strains present a more adenomatous type of growth than the three others, which are predominantly alveolar in structure. As previously shown (1), the rate of oxygen consumption by these tumours falls with diminishing speed of growth, exception being made for the slowly growing adeno-carcinomata where there is a rise above what might be expected.

In assessing the rate of growth of different tumour strains, the outlines of the tumours produced at intervals of one or two weeks have been compared, but this is at best only a rough comparison. The actual increment is the resultant of two opposing factors—the multiplication of cells on the one hand, and the death of many cells on the other. The visible speed of growth need not therefore represent the actual rate of multiplication of the tumour-cells, for the rate of necrosis may vary considerably, and obscure the varying production of new cells by different parenchymata. Only pronounced differences in the visible rate of growth of different tumour strains can be regarded as significant.

TABLE V.—*Sarcoma* 37 *S.*

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·936	7·6	201	460
0·942	7·0	180	,,
0·901	5·9	395	800
0·948	7·0	201	460
0·976	5·4	188	,,
0·946	12·0	150	,,

Average respiratory quotient 0·941, ± 0·010.

37 S. is a very rapidly growing spindle-celled sarcoma which arose during

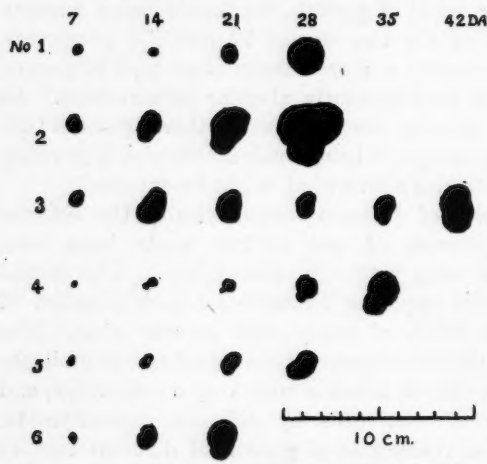
the transplantation of a mammary adeno-carcinoma in the manner described by Haaland (4). On inoculation it grows in a high percentage of animals, but many of the tumours later recede and disappear.

TABLE VI.—*Carcinoma* 206.

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·916	5·3	144	460
1·000	8·2	153	,,
0·795	5·9	302	800
0·933	3·5	202	460
0·810	5·2	255	800
0·753	4·2	306	,,

Average respiratory quotient 0·868, ± 0·039.

Carcinoma 206 is an alveolar carcinoma of the mamma. The transplanted growths are extremely prone to undergo spontaneous absorption, and induce a highly refractory state to subsequent inoculation of the mice (5). The rate of growth of individual tumours varies extremely in this strain; sometimes it will grow even faster than 37 S., but at others it will slow up very much. On Text-fig. 1 are given the outlines at weekly intervals of the six tumours used in the respiration experiments in the same order. From this it will be seen that there is a tendency for the respiratory quotient to rise with increasing speed of growth.



TEXT-FIG. 1.—Chart showing rate of growth of the six tumours of carcinoma 206 tested.

TABLE VII.—*Carcinoma* 63.

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·853	7·9	154	460
0·794	8·0	145	,,
0·841	5·4	186	400
0·854	7·2	164	460
0·771	6·2	259	800
0·798	6·4	280	,,

Average respiratory quotient 0·818, ± 0·014.

Carcinoma 63 is predominantly alveolar in structure. It grows more

slowly than the two previous tumour strains, but is much steadier in its behaviour.

TABLE VIII.—*Carcinoma* 113.

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·924	4·7	296	800
0·980	4·6	298	,,
0·948	2·9	211	,,
0·901	4·2	204	,,
1·000	4·3	327	,,

Average respiratory quotient 0·951, ± 0·018.

This tumour is also an alveolar carcinoma and grows much more slowly than carcinoma 63. Its special feature, however, is the constant presence of considerable quantities of glycogen as shown in Plate-fig. 2. The left-hand portion represents a fragment of the emulsion before incubation, and the right-hand a similar portion after one and a half hours' incubation. In this time no appreciable diminution could be detected by the microscope in the amount of glycogen present. This is scarcely what might have been expected from the very high respiratory quotient given by all the five samples. If the microscopic behaviour of the glycogen in the liver and this tumour be compared with the respective quotients found there is a pronounced discordance. The extremely fine character of the glycogen granules in the liver-cells will of course facilitate the process of glycogenolysis, whether the carbohydrate be needed for immediate combustion or not.

TABLE IX.—*Carcinoma* 72.

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·629	4·1	221	800
0·837	4·0	317	,,
0·649	3·2	310	,,
0·714	4·7	283	,,
0·732	4·3	307	,,
0·752	3·9	278	,,

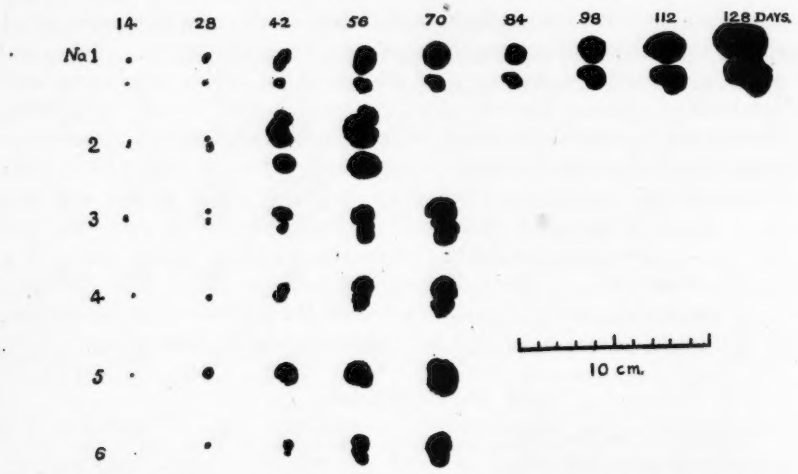
Average respiratory quotient 0·719, ± 0·031.

TABLE X.—*Carcinoma* 155.

Respiratory quotient.	Oxygen in c.c. per kgrm. min.	Quantity in mgrm.	Surface in sq. mm.
0·683	6·5	330	800
0·661	4·8	351	,,
0·706	5·3	143	460
0·697	4·0	253	800
0·617	4·7	306	,,
0·678	4·8	286	,,

Average respiratory quotient 0·674, ± 0·013.

Carcinoma 72 and carcinoma 155 are very similar in structure and type of growth. The general type of structure is predominantly acinous, the acini being admittedly imperfectly constructed, with the lumen often drawn out into a long slit. Both grow extremely slowly, carcinoma 155 being even slower than 72. Text-fig. 2 shows at fortnightly intervals the silhouettes of the six tumours of 72 which were tested, and it will be seen that the unusually rapid growth of the second specimen gave a much higher respiratory quotient than the other five. The abnormally low quotients given by 155 are possibly due to imperfection in the method used, although they may be produced by an incomplete oxidation of the intermediate products of katabolism.



TEXT-FIG. 2.—Chart showing rate of growth of the six tumours of adeno-carcinoma 72 tested.

An epithelioma which started on the back of a male mouse and which is being successfully transplanted, gave on the three occasions tested respiratory quotients of 0·846, 0·755 and 0·862. In rate of growth this tumour equals carcinoma 113, but its excessive tendency to undergo spontaneous absorption and to keratinise renders the securing of suitable material difficult. Only one spontaneous tumour has been examined and the quotient 0·785 found. The tumour was a hæmorrhagic adeno-carcinoma of the breast, and the material tested came from a fairly rapidly growing mass which recurred after operation.

The above average respiratory quotients show greater differences in the case of the tumour tissues than in the case of the four normal parenchymata tested, and the differences in the case of the tumours present a hitherto unsuspected feature. The consistency of the results obtained with the

individual tissues inspires a certain degree of confidence, but as they are the first attempt to fix a respiratory quotient for different mouse tissues, and as the method of analysis is admittedly defective, caution in interpreting the results is indicated. Although much experimental work has been done on the oxygen consumption of organs and tissues, there are few cases where a consistent respiratory quotient has been established for surviving tissues.

Vernon (6) obtained a quotient of 0·85 for the rabbit kidney perfused with oxygenated Ringer at room temperature. This is fairly close to the figure found by us for mouse kidney.

Cohnheim and Pletnew (7) found for the cat's intestine a quotient from 1·07 up to 1·29, but their method involved the destruction of the lining epithelium and bacteria by a 0·17 per cent. solution of corrosive sublimate. In a large series of experiments on the heart-lung preparation from dogs, Starling and Evans (8) obtained a quotient of 0·85 for the normal dog. The hind limbs of a dog were found by von Frey and Gruber (9) to give a quotient slightly above unity. Battelli and Stern (17), who worked with minced tissues of the dog, found for the heart and liver quotients of 0·72 and 0·58 respectively. Harden and Maclean (10) obtained quotients varying from 0·9 to 1·3 with minced rabbit muscle. Rabbit liver was investigated by Fedeli (18), and a quotient of 0·725 ascertained.

In addition to the four normal tissues a few experiments were carried out by us on embryonic tissues, which share with tumours a rapid proliferation rate. Three were done on emulsions of the total embryo, and gave quotients varying from 1·3 to 1·1, but the rate at which the excess of carbon dioxide came off dropped rapidly during the first thirty or forty minutes of the incubation. The abnormality of the quotient with this tissue is probably due to the lactic acid formation in the muscle cells driving out carbon dioxide from pre-existent combination. Fletcher (11) has shown clearly, both for minced rabbit and rat muscle, that there is a rapid rise in the amount of lactic acid present during the first half hour, and an excessive evolution of carbon dioxide in the same period. On two occasions the respiratory exchange of an emulsion made from mouse embryo skin was tested, and on each occasion a quotient of unity was found. The experiments on embryonic tissues have been hampered by an unusual run of sterility in our breeding stock, and will have to be pursued as occasion offers.

Whilst many observations have been made upon the respiratory exchange of the embryos of cold-blooded animals and upon the developing eggs of hens, but few attempts have been made with warm-blooded animals. Cohnstein and Zuntz (12) made the attempt on sheep embryos, but did not succeed in establishing a respiratory quotient. Bohr (13) had more success with the guinea-pig, and concluded that the total gas exchange was about the same as for the mother with a respiratory exchange about unity. His figures, however, show wide variations in the different experiments. That a developing mammal should

have a respiratory quotient about unity stands in marked contrast with the low quotient of 0·71 found in the developing chick [14], for the former means pure carbohydrate metabolism and the latter a pure fat metabolism.

It was already noted that the quotient of the mamma of pregnant mice was apparently higher about the sixteenth day than at later stages of gestation. This fall might be correlated with the establishment of lactation, the metabolic exchange of the gland passing from that of pure development to one of functional activity. Before the onset of the latter the gland would appear to be burning carbohydrate only. Kidney, liver and salivary gland all have quotients pointing to a mixed metabolism, but in default of any estimate of the probable nitrogenous metabolism of the mouse, it scarcely seems feasible to arrive at approximate figures for the respective amounts of food-substances which would give the various quotients. In any case the imperfections of the method of arriving at the respiratory quotient do not justify us in drawing any but the broadest and most apparent conclusions.

It may be here recalled that Cramer [15], in comparing the gaseous exchange of rats bearing inoculated tumours with normal rats, found no essential difference in the respiratory quotient. Chisolm [16] came to a similar result in the case of mice. The relatively small part which a necrosing tumour occupies in the total gaseous metabolism of the animal might readily obscure such differences as we have found in the observations detailed above.

In general it may be stated that when one tumour strain is compared with another there is, with increasing rate of growth, a rise in the respiratory quotient. The glycogen-rich tumour 113 makes an important exception to this statement, for although its rate of growth is slow, yet it gives the highest average quotient found for neoplastic tissues. Its large store of intracellular glycogen gives it a superabundant supply of carbohydrate to draw upon, although there is scarcely any histological evidence of reduction in the amount of glycogen after one and a half hours' incubation. For the greater part of this period, however, the tumour may, like the kidney, be drawing upon invisible carbohydrate. The storing of glycogen by this tumour parenchyma may be looked upon as a pathological excess over its actual needs.

Microscopic examination of the different tumour emulsions has shown that all contain plenty of fatty material, but it is only the very slowly-growing tumours 72 and 155 which give a quotient representing the exclusive combustion of fats. An interesting confirmation of the fat metabolism prevailing in these two strains was obtained by comparing the survival of the mitochondria. Whereas the mitochondria of the other tumours and normal tissues were well preserved after one and a half hours' incubation, in these two strains they underwent a rapid degeneration and disappeared. The histological evidence of this change will be dealt with in a later communication by one of us (B.R.G.R.).

In conclusion, attention may be drawn to the circumstances that these experiments do not deal with the respective part played by different food-substances participating in the building-up of a given tumour parenchyma, but only bear upon the sources from which the tumour derives the necessary energy. The distribution of this energy between energy of growth and energy of function is at present impossible in view of the elusive character of function in tumours. In so far as a conclusion may be drawn, the experiments do not go further than showing that rapidly-growing tumours draw more on carbohydrates for their energy, whilst slowly-growing more differentiated tumours apply themselves to fats.

SUMMARY.

Respiratory quotients were obtained for surviving normal and tumour tissues of the mouse by suspending emulsions in alveolar air in the Barcroft blood-gas apparatus. The results agreed very closely in successive experiments with each normal tissue. Greater variability was found with tumours:

Normal.		Rate of growth ↑ Differentiation ↓	Tumour.
Kidney	0·883 ± 0·002	37 S.	0·941 ± 0·010
Liver	0·813 ± 0·024	206	0·868 ± 0·039
Mamma	0·914 ± 0·027	63	0·818 ± 0·014
Submaxillary gland	0·841 ± 0·024	113	0·951 ± 0·018
(Total embryo	1·3—1·1)	72	0·719 ± 0·031
(Mouse embryo skin	1·0)	155	0·674 ± 0·013

The tentative conclusion is drawn that the more rapidly-growing, undifferentiated tumours draw their energy from carbohydrate, the more slowly-growing from fats.

REFERENCES.

1. Russell, B. R. G., and Gye, W. E.—"The Oxygen Consumption of Normal and Cancerous Mouse Tissues *in vitro*," *Brit. J. Exper. Pathol.*, 1920, **1**, 175.
2. Krogh, A.—"Ein Mikrorespirationsapparat, etc.," *Biochem. Zeitschr.*, 1914, **62**, 266, and **66**, 512.
3. Hoffman, P.—"A Simple Method of Calibrating the Differential Blood-gas Apparatus," *J. Physiol.*, 1913, **47**, 272.
4. Haaland, M.—"Contributions to the Study of the Development of Sarcoma under Experimental Conditions," 'Third Sci. Rep. Imp. Cancer Res. Fund,' London. 1908 (Taylor & Francis), p. 175.
5. Russell, B. R. G.—"The Manifestation of Active Resistance to the Growth of Implanted Cancer," 'Fifth Sci. Rep. Imp. Cancer Res. Fund,' London, 1912 (Taylor & Francis), p. 1.
6. Vernon, H. M.—"The Conditions of Tissue Respiration," *J. Physiol.*, 1906, **35**, 53
7. Cohnheim, O., and Pletnew, D.—"Der Gaswechsel der Muskulatur des Dünndarms," *Zeitschr. f. physiol. Chemie.*, 1910, **69**, 96.

8. Starling, E. H., and Evans, C. L.—"The Respiratory Exchanges of the Heart in the Diabetic Animal," *J. Physiol.*, 1914, **49**, 67.
9. von Frey, M., and Gruber, M.—"Untersuchungen über den Stoffwechsel isolierter Organe," *Arch. f. Physiol.*, 1885, **9**, 519.
10. Harden, A., and Maclean, H.—"The Oxidation of Isolated Animal Tissues," *J. Physiol.*, 1911, **43**, 34.
11. Fletcher, W. M.—"Lactic Acid Formation, Survival Respiration, and *Rigor Mortis* in Mammalian Muscle," *ibid.*, 1913, **47**, 361.
12. Cohnstein, J., and Zuntz, N.—"Untersuchungen über das Blut, den Kreislauf, und die Atmung beim Säugethier-Fötus," *Arch. f. d. ges. Physiol.*, 1884, **34**, 173.
13. Bohr, Chr.—"Der respiratorische Stoffwechsel des Säugetierembryo," *Skand. Arch. f. Physiol.*, 1900, **10**, 413.
14. Bohr, Chr., and Hasselbalch, K. A.—"Ueber die Wärmeproduktion und den Stoffwechsel des Embryos," *ibid.*, 1903, **14**, 396.
15. Cramer, W.—"The Gaseous Metabolism in Rats Inoculated with Malignant New Growths," 'Third Sci. Rep. Imp. Cancer Res. Fund,' London, 1908 (Taylor & Francis), p. 427.
16. Chisolm, R. A.—"The Respiratory Exchange of Mice bearing Transplanted Carcinoma," *J. Pathol. and Bacteriol.*, 1911, **15**, 192.
17. Battelli, F., and Stern, L.—"Recherches sur la respiration élémentaire des tissus," *J. de physiol. et pathol. gén.*, 1907, **9**, 1, 34 and 228; *Compt. rend. Soc. de biol.*, 1907, **62**, 296, 386, 531, 596, 832, 958 and 1110.
18. Fedeli, A.—"De la respiration du sang et des tissus chez les animaux néphrectomisés," *J. de physiol. et pathol. gén.*, 1913, **15**, 276.

ERRATUM.

In Dr. Cramer's paper, "On Glandular Adipose Tissue," page 190, line 19, "*on* material" should read "*no* material."

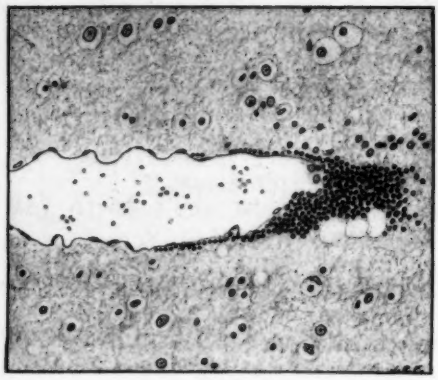


Fig. 1.
Mac. cynomolgus. Spontaneous infection. Haem. and eosin. Obj. $\frac{1}{6}$". Oc. 4 Zeiss.

Fig. 2.
Mac. cynomolgus. Passage I. Sacral cord. Haem. and eosin. Obj. $\frac{1}{6}$". Oc. 4 Zeiss.

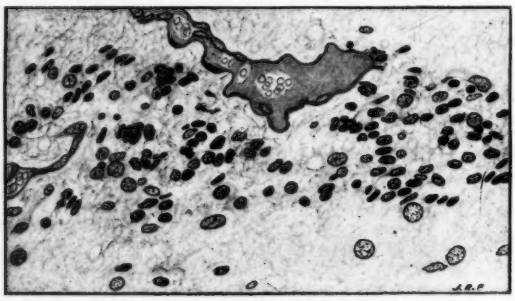


Fig. 3.
Mac. cynomolgus. Passage I. Rt. basal ganglia. Haem. and eosin. Obj. $\frac{1}{6}$" Oc. 4 Zeiss.

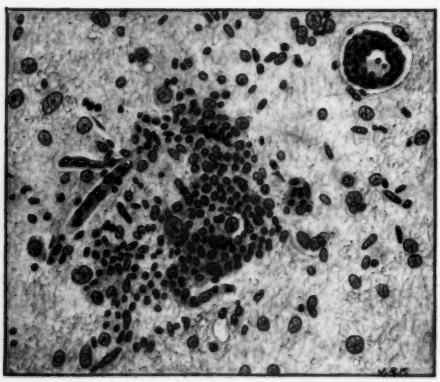


Fig. 4.
Rabbit. Passage I. Frontal lobe. Haem. and eosin. Obj. $\frac{1}{6}$". Oc. 4 Zeiss.

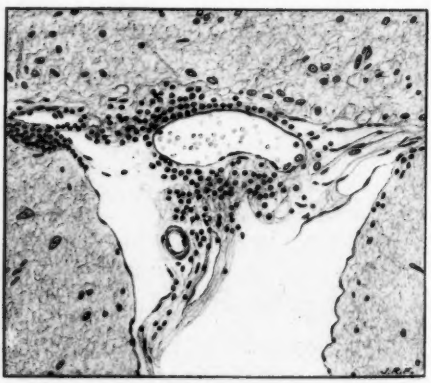


Fig. 5.
Rabbit. Passage I. Mid-brain. Haem. and eosin. Obj. $\frac{1}{6}$". Oc. 4 Zeiss.

TRANSMISSION OF EXPERIMENTAL *ENCEPHALITIS LETHARGICA* IN SERIES IN MONKEYS AND RABBITS, WITH NOTES ON A SPONTANEOUS INFECTION IN A MONKEY.*

JAMES McINTOSH,

From the Bland Sutton Institute of Pathology, Middlesex Hospital, London.

Received for publication September 23rd, 1920.

In a previous number of this Journal (1) an account was given of the successful transmission of *encephalitis lethargica* to a Patas monkey. The present article deals with the transmission of the disease in series—a detail necessary to complete the proof that the disease is caused by a living virus. At the same time an account is given of a spontaneous infection in another monkey.

PASSAGE I.

The virus was obtained from the successfully inoculated Patas monkey. Small pieces of the cervical cord, pons, basal ganglia and cortex were crushed in a conical glass vessel with a glass rod and then emulsified in 20 c.c. of sterile normal saline. The supernatant fluid was then drawn into a syringe and inoculated into two *Macacus cynomolgus* monkeys. Each animal received 1 c.c. of the emulsion intracerebrally and 5 c.c. subcutaneously.

Symptoms and Lesions of the Experimental Animals.

A. *Macacus cynomolgus* No. 1. Large male.

28/11/19.—Inoculated.
14/1/20.—No abnormal symptoms have been noticed.
10/2/20.—Animal quite fit.
12/2/20.—Reinoculated with the same virus.
12/3/20.—No abnormal symptoms have appeared to date.
14/5/20.—Ditto.

B. *Macacus cynomolgus* No. 2. Female.

28/11/19.—Inoculated.
14/1/20.—Quite healthy; no abnormal signs so far.
12/2/20.—Reinoculated.
16/2/20.—Quite fit.

* The expenses of this research were defrayed by the Medical Research Council.

23/2/20.—Has a cold—running at the nose, etc.

11/3/20.—Does not appear to be quite well; some trembling of arms and tendency to shake and rotate its head.

12/3/20.—Condition similar. This monkey, which was previously very playful, now refuses to play. Stumbles when walking on the floor, and its whole gait is clumsy.

13/3/20.—More lively; seems rather better and eats well.

15/3/20.—Quieter again to-day; will not play. There is a slight conjunctivitis of right eye, and the animal is very thirsty.

17/3/20.—Condition similar.

22/3/20.—Seems worse to-day; still has slight cold in the head. The animal might be described as being slightly somnolent; keeps eyes shut most of the time. When roused is querulous. Appears to have difficulty in swallowing and stretches out its neck while drinking.

23/3/20.—Seems slightly better.

27/3/20.—Condition worse again; still difficulty in swallowing.

31/3/20.—Hind legs are now weak and it can no longer stand erect as it used to. Twitches its head.

1/4/20.—Condition similar.

11/4/20.—This morning the animal was noticed to be very excited and jumping about in its cage. Soon after this it cried out and had a severe epileptiform fit, which lasted about an hour. It lay on its back moving its arms and legs in a purposeless manner. The eyes were open and the tongue extended and directed a little to the right. Later on the limbs became rigid and tremulous. The condition then gradually passed off, although the animal stumbled about for some time after. By the afternoon the animal had recovered considerably, but it was very weak and could not climb back into the cage.

12/4/20.—Condition improved. Could climb back into cage; still difficulty in drinking.

14/4/20.—Condition as above.

19/4/20.—Much weaker and stumbles a lot.

21/4/20.—Weaker. Killed by coal-gas.

Autopsy.—Apart from a distinct dryness and almost complete absence of fat in the subcutaneous tissues, nothing abnormal was observed in any of the internal organs. Macroscopically sections of the brain showed congestion of the grey matter. Pieces of the brain were placed in 33 per cent. glycerine for preserving the virus, while other portions were fixed in 10 per cent. formalin-saline for histological purposes.

Histological Examination of the Brain of Macacus cynomolgus *No.* 2.

Left frontal lobe.—Meninges in places show a slight increase of cellular elements, chiefly lymphocytes. The vessels of the cerebral substance are

congested and often contain hyaline thrombi. The pyramidal cells appear normal.

Left parietal lobe.—In the cerebral substance there are a few small areas of perivascular infiltration, consisting chiefly of glial cells, lymphocytes, and a larger endothelial type of cell.

Left occipital lobe.—In one area there is a distinct perivascular small-celled infiltration and numerous hyaline thrombi.

Right basal ganglia—anterior.—Ganglion cells well formed; Nissl granules prominent.

Right basal ganglia—lenticular nucleus.—In this region the ganglion cells are frequently degenerate and shrunken; the nuclei often eccentric and staining deeply.

Right basal ganglia—posterior.—Condition as above; vessels frequently contain hyaline thrombi.

Section through caudate nucleus—left.—Vessels dilated, numerous hyaline thrombi; multiple hæmorrhages into the perivascular sheaths and surrounding tissues. In places distinct glial proliferation. Ganglion cells degenerate and often surrounded by glial cells.

Section through upper portion of corpora quadrigemina.—Many degenerate ganglion cells in lower part; the degenerate cells are to be found alongside healthy cells which have well-marked Nissl granules. There are numerous hæmorrhages to be seen in this section.

Section through pons at level of fifth root.—In many of the ganglion cells the Nissl granules are distinct, but among them are to be seen what appear to be degenerate cells. The latter show eccentric nuclei and chromatolysis. In some cases only the nuclei are to be seen surrounded by small mononuclear cells. Glial proliferation is quite distinct.

Upper cervical cord.—In the meninges nothing abnormal made out. Ganglion cells mostly in good condition with granules well marked.

Central cervical.—As above, but a few small hæmorrhages present in both horns.

Upper and lower thoracic.—Nothing abnormal seen.

Lumbar cord.—Vessels congested.

Sacral cord.—Many degenerate ganglion cells in both horns with close application of proliferated glial cells. In certain instances as many as six to eight such nuclei could be counted.

Acute inflammatory changes were therefore observed within the nervous system. The lesions consisted chiefly of glial proliferation, slight perivascular infiltrations of glial cells and lymphocytes. In addition there were congestion, hyaline thrombi and multiple hæmorrhages into the perivascular sheaths and surrounding tissue.

The degenerative changes affected mainly the ganglion cells, and consisted of chromatolysis, vacuolation of the protoplasm and displacing of the

nucleus. Other cells again were shrunken and stained deeply. The degeneration of these cells was such as to suggest that it had occurred during the life of the animal. In fact the short time that was allowed to elapse between the death of the animal and the fixing of the tissues almost excludes the possibility of any *post-mortem* change.

The general histological appearance shows a close similarity to those found in the first successfully inoculated monkey. One or two minor differences were observed, but they were rather of degree than of kind. The changes, on the whole, appeared to be earlier and therefore tended to be more general, in that the inflammatory lesions, although in relation to the blood-vessels, seldom consisted of dense perivascular accumulations. In addition, degeneration changes in the ganglion cells of the cord, particularly in the lower parts, were much more apparent. The affection of the cord, although in no way comparable to that found in experimental poliomyelitis, is of considerable interest. But before one can make definite statements on the relationship of this disease to poliomyelitis, a lot of work will have to be done. So far all experiments, serological and otherwise, tend to prove that the two viruses are distinct.

PASSAGE II.

Pieces of the spinal cord and brain (including basal ganglia) obtained at the autopsy of the *Macacus cynomolgus* monkey No. 1 were directly emulsified in normal saline, and inoculated under the dura and subcutaneously into two monkeys and three rabbits. The following results were observed:

A. Young Male Baboon.

21/4/20.—Inoculation.

22/4/20.—Quite recovered from operation.

26/4/20.—Quite fit.

29/4/20.—Ditto.

1/5/20.—Appears to be out of sorts, hair roughened and animal jumpy.

The above condition persisted for a week and then passed off.

15/8/20.—Animal noticed to be out of sorts. It gradually became weaker. The arms were noticed to be tremulous, and it had difficulty in picking things up with its fingers. No distinct paralysis was observed. The animal was described as dull and apathetic.

1/9/20.—In the afternoon the monkey had a well-marked fit which gradually passed off. Animal gradually became weaker and died after a fit on September 3rd.

Autopsy.—The autopsy was performed by Dr. Woods, who found nothing apart from some wasting of the tissues. Pieces of the brain and cord were placed in 50 per cent. glycerine for inoculation purposes, and the remaining portions were preserved for histological examination.

B. *Cercopithecus callitrichus.*

21/4/20.—Inoculated, but never showed the least abnormal sign, although kept under observation for over four months.

C. Rabbits.

21/4/20.—Three rabbits inoculated; each received 0·25 c.c. intra-cerebrally and 2·5 c.c. subcutaneously.

22/4/20.—Animals fit and eat well.

23/4/20.—Ditto.

24/4/20.—Black rabbit rotates head from side to side.

26/4/20.—Black and grey rabbits out of sorts and stumbling in their gait. Temperature, brown 103·4° F., black 105·5° F. Both are very excited when touched.

27/4/20.—Brown rabbit has well-marked choreiform movements.

29/4/20.—Black rabbit very ill, more or less paretic, respiration slowed. Animal killed.

Autopsy.—Autopsy showed no macroscopic changes in the brain or internal organs. The cerebro-spinal fluid was clear and contained only a few lymphocytes. Pieces of the brain and spinal cord were fixed in formalin for histological purposes, and other portions were emulsified in saline and inoculated intra-cerebrally into three other rabbits, one of which developed similar symptoms.

The choreiform movements in the brown rabbit persisted for some time and then more or less completely passed off. The third rabbit never showed any abnormal signs.

Histological Changes in Rabbit's Brain and Cord.

Section through fore-brain shows a slight infiltration of the meninges with lymphocytes. In the cortex one or two small cellular infiltrations are seen. The ganglion cells seem mostly normal.

Section through mid-brain, including basal ganglia.—Meninges show a slight increase of lymphocytes. Inflammatory foci in the brain-substance not infrequent, consisting of mononuclear cells—lymphocytes and glial cells. The small vessels frequently have an infiltration of small cells along the sheath.

Upper cervical cord.—Nothing abnormal seen.

SPONTANEOUS INFECTION IN A *MACACUS CYNOMOLGUS.*

It is interesting to record here that a *Macacus cynomolgus* monkey kept as a control along with an inoculated monkey developed symptoms identical with those which the inoculated animal showed.

Three monkeys (*Macacus cynomolgus*) were obtained at the same time. On November 28th, 1919, two of these (a large male and a small female) were inoculated with the virus of the first passage. The third animal, a small male, was kept as the control. As neither of the two inoculated animals showed any sign of illness they were re-inoculated with the same virus on February 12th, 1920, the small female later on developing the infection in a typical manner. On February 14th, 1920, the small uninoculated male was observed to have a fit, although previous to this he seemed quite healthy. The animal then gradually became weaker, had one or two more fits, and died on February 21st, 1920. At the autopsy the brain was found to be slightly congested, but the other internal organs were quite healthy and nothing to account for death was detected.

A histological examination of the basal ganglia of the brain demonstrated the presence of inflammatory lesions such as we have become accustomed to consider as characteristic of *encephalitis lethargica*. These consisted of perivascular accumulation of lymphocytes and glial cells.

The fits, the absence of any gross lesion to account for the death of the animal, along with the characteristic histological changes found in the brain, justify one in regarding this as a spontaneous infection of encephalitis in a monkey. The period of time which elapsed between the death of the animal and the first inoculation of the companion monkeys was eighty-five days; it is quite likely, however, that the animals are infectious some considerable time before any symptoms develop as is the case with poliomyelitis. Owing to the impossibility of obtaining other monkeys at this period, inoculation experiments were not carried out.

CONCLUSIONS.

The successful transmission in series of experimental *encephalitis lethargica* to monkeys and rabbits has completed the experimental proof necessary to show that the disease is caused by a living virus. A similar result has been obtained by Levaditi and Harvier (2). The spontaneous case recorded above is additional proof. In the second passage the inflammatory lesion was not quite so strictly localised. This is apparently due either to some change in the virus or to the lesion not being so advanced. The greater involvement of the cord is in keeping with the more diffuse nature of the brain lesion.

In conclusion, I wish to express my indebtedness to Dr. Turnbull and to Dr. W. W. Woods for their valuable assistance in many directions.

REFERENCES.

1. McINTOSH, J., AND TURNBULL, H. M.—"The Experimental Transmission of *encephalitis lethargica* to a Monkey," *Brit. J. Exper. Pathol.*, 1920, **1**, 89.
2. LEVADITI, C., AND HARVIER, P.—"Recherches expérimentales sur l'encéphalite léthargique," *Compt. rend. Soc. de biol.*, 1920, **83**, 674.